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Isolation and characterization of antiplasmodial compounds from *Siphonochilus aethiopicus* and *Aloe ferox* and bioavailability of a novel furanoterpenoid

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**Doctor of Philosophy in Pharmacology**

Department of Medicine

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## DECLARATION

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## Abstract

Malaria is the most important vector-borne disease mainly affecting sub-Saharan Africa. Malaria, like tuberculosis, is curable but the disease is most prevalent in populations, which cannot afford modern pharmaceuticals. The malaria parasite has developed resistance to most antimalarial drugs. Medicinal plants have been used since ancient times to treat a variety of diseases. Traditional healers are more common in developing countries than medical doctors and more than 50% of the South African population consults traditional healers. The aim of this study was to investigate two medicinal plants *Siphonochilus aethiopicus* (Zingiberaceae) and *Aloe ferox* (Asphodelaceae) for their antimalarial properties and to isolate and characterize novel antiplasmodial compounds. Preliminary bioavailability studies of a novel furanoterpenoid isolated from *S. aethiopicus* were performed.

The ethyl acetate extract of *S. aethiopicus* was active *in vitro* against both chloroquine (CQ) sensitive D10 and CQ resistant K1 strains of *P. falciparum* with IC<sub>50</sub>-values of 2.9 µg/ml and 1.4 µg/ml, respectively. This extract was subjected to bioassay-guided fractionation which yielded three novel structurally-related furanoterpenoids. Nuclear magnetic resonance (1D and 2D) and mass spectrometry was used to determine the chemical structure of compounds. The most active furanoterpenoid (C<sub>15</sub>H<sub>16</sub>O<sub>3</sub>) showed similar *in vitro* antiplasmodial activity to the ethyl acetate extract. No *in vitro* cytotoxicity was observed with the ethyl acetate extract as well as the isolated compounds against the Chinese Hamster Ovarian cell-line. The ethyl acetate extract and compounds isolated were more active *in vitro* against the CQ resistant strain of the parasite compared to the CQ sensitive strain. *In vivo* testing was performed using a murine malaria model. The ethyl acetate extract of *S. aethiopicus* was more active *in vivo* than the most active compound using a 4-day suppressive test and showed no toxicity. The *in vivo* schizontocidal activity of the *S. aethiopicus* extract was further investigated in a 7-day suppressive test using two drug formulations; an aqueous-based 5% sodium bicarbonate (with addition of 5% ethanol) and lipid-based formulation. The 5% sodium bicarbonate formulation resulted in a suppression of parasitemia while the lipid-based formulation did not suppress parasitemia. Further *in vivo* testing of the ethyl acetate extract did not reveal parasite clearance and a weak to

moderate prophylactic effect was observed. Drug formulation and route of administration have an effect on pharmacokinetic factors such as bioavailability and rate of metabolism resulting in either increased or decreased *in vivo* schizontocidal activity. The active compound might be metabolized to an inactive component or be poorly absorbed. An *in vivo* mouse-model was developed to investigate pharmacokinetic parameters. Bioavailability studies of the most active compound showed that formulation and route of administration have an effect on drug absorption. Thus *in vivo* schizontocidal activity might be improved if given in optimal formulation and route of administration. The absorption of the active component was improved when administered as a crude extract as opposed to a single entity. A preliminary *in vivo* metabolism study could not identify the metabolites of the active compound. The pharmacokinetic study suggests that the lack of *in vivo* schizontocidal activity of the active furanoterpenoid is due to poor absorption rather than rapid metabolism.

The dichloromethane extract of *A. ferox* whole leaf collected in winter were more active *in vitro* against *P. falciparum* than the summer collections. The whole leaf extracts showed greater antiparasmodial activity than the inner gel and outer leaf separated. Four compounds were isolated from the whole leaf extract and outer leaf extract through bioassay-guided fractionation. Two known compounds were isolated namely aloe-emodin and aloesin as well as two unknown compounds. The most active compound was moderately active against the CQ sensitive strain of *P. falciparum* with an  $IC_{50}$ -value of 2.9  $\mu\text{g/ml}$ . The exact chemical structure could not be determined. The other three compounds were not active *in vitro*. The investigation of whole leaf dichloromethane extract of *A. ferox* for *in vivo* schizontocidal activity revealed greater suppression of parasitemia against the CQ resistant strain than the CQ sensitive strain of the parasite. No toxicity was observed with the crude extract during *in vitro* or *in vivo* testing. Different components in *A. ferox* might play a role in the observed antiparasmodial activity.

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## List of Abbreviations

<b>AA</b>	Acetic acid (glacial)
<b>ACN</b>	Acetonitrile
<b>AE</b>	Aloe-emodin
<b>APAD</b>	3-acetylpyridine adenine dinucleotide
<b>API</b>	Atmospheric pressure chemical ionization
<b>ART</b>	Artemisinin
<b>ATCC</b>	American Type Culture collection
<b>ATP</b>	Adenosine triphosphate
<b><sup>13</sup>C</b>	Carbon 13
<b>CC</b>	Column chromatography
<b>CDC</b>	Centres for Disease Control and Prevention
<b>CHO</b>	Chinese hamster ovarian
<b>CIP</b>	Pasteur Institute Culture collection
<b>CM</b>	Complete medium
<b>COSY</b>	Correlation spectroscopy
<b>CQ</b>	Chloroquine
<b>CQR</b>	Chloroquine-resistant
<b>CQS</b>	Chloroquine-sensitive
<b>CYP</b>	Cytochrome P450
<b>1D</b>	One dimensional
<b>2D</b>	Two dimensional
<b>DCM</b>	Dichloromethane
<b>DDT</b>	Dichloro-diphenyl-trichloroethane
<b>dH<sub>2</sub>O</b>	Distilled water
<b>DHFR</b>	Dihydrofolate reductase
<b>DHPS</b>	Dihydropteroate synthetase
<b>DMEM</b>	Dulbecos modified eagles medium
<b>DMSO</b>	Dimethyl sulfoxide
<b>DNA</b>	Deoxyribonucleic acid
<b>EA</b>	Ethyl acetate
<b>ESI</b>	Electrospray Ionisation
<b>FCS</b>	Foetal calf serum
<b>GI</b>	Growth index
<b><sup>1</sup>H</b>	Proton
<b>HAMS</b>	HAM's Nutrient mixture F-12
<b>HEPES</b>	N-[2-Hydroxyethyl]-piperazine-N'-[2-Ethansulphonic acid]
<b>Hex</b>	Hexane
<b>HIV</b>	Human immunodeficiency virus
<b>HMQC</b>	Heteronuclear multiple quantum correlation
<b>HPLC</b>	High performance liquid chromatography
<b>HR-APCI-MS</b>	High resolution atmospheric pressure chemical ionization mass spectrometry
<b>HSQC</b>	Heteronuclear single quantum correlation
<b>IC<sub>50</sub></b>	Concentration inhibiting 50% of growth
<b>IL-6</b>	Interleukin-6
<b>INT</b>	p-iodonitrotetrazolium



<b>J</b>	Coupling constant
<b>LC-MS</b>	Liquid chromatography mass spectrometry
<b>LC-MS/MS</b>	Liquid chromatography mass spectrometry/mass spectrometry
<b>LDH</b>	Lactate dehydrogenase
<b>LLOQ</b>	Lower limit of quantification
<b>MeOH</b>	Methanol
<b>mH<sub>2</sub>O</b>	Millipore water
<b>MIC</b>	Minimum inhibitory concentration
<b>MP</b>	Mobile phase
<b>MQ</b>	Mefloquine
<b>MRM</b>	Multi reaction monitoring
<b>MS</b>	Mass spectrometer
<b>MTT</b>	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
<b>NAD</b>	Nicotinamide adenine dinucleotide
<b>NBT</b>	Nitro blue tetrazolium
<b>ND</b>	Not determined
<b>NHLS</b>	National Health Laboratory Service
<b>NMR</b>	Nuclear magnetic resonance
<b>NOESY</b>	Nuclear Overhauser enhancement and exchange spectroscopy
<b>PBS</b>	Phosphate buffered saline
<b>PE</b>	Petroleum ether
<b>P-gp</b>	P-glycoprotein
<b>pLDH</b>	Parasite lactate dehydrogenase
<b>pRBC</b>	Parasitized red blood cells
<b>PTLC</b>	Preparative thin layer chromatography
<b>PYR</b>	Pyrimethamine
<b>Q1</b>	Quadrupole 1
<b>Q3</b>	Quadrupole 3
<b>Q-TOF</b>	Time of flight mass spectrometer
<b>RBC</b>	Red blood cell
<b>RI</b>	Resistance index
<b>RP</b>	Reverse phase
<b>SB</b>	Sodium bicarbonate
<b>SC</b>	Subcutaneous
<b>SD</b>	Standard deviation
<b>SDA</b>	Sabouraud dextrose agar
<b>SFD</b>	Sulfadoxine
<b>SI</b>	Selectivity index
<b>SMEDDS</b>	Self microemulsifying drug delivery system
<b>SPE</b>	Solid phase extraction
<b>TB</b>	Tuberculosis
<b>TLC</b>	Thin layer chromatography
<b>TM</b>	Traditional medicine
<b>TNF</b>	Tumour necrosis factor
<b>TSA</b>	Tryptone soya agar
<b>TSB</b>	Tryptone soya broth
<b>UV</b>	Ultraviolet
<b>WHO</b>	World Health Organisation

# *Chapter 1*

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## *Introduction*

University of Cape Town

## 1. Introduction

### 1.1 History of malaria

Malaria is an ancient disease still affecting modern society. Records of malaria incidences date back to 6000 years in Egypt and China and 1600 BC in India (WHO, 1986). The Ancient Greeks and Romans have made the association between fevers and stagnant waters and swamps (Konradsen et al., 2004). Soon after the discovery of mosquitoes as the vectors of malaria various interventions were implemented to eradicate this disease. Kerosene oil was applied to potential breeding sites but this had a limited effect due to the oil evaporating in the warm climate. Canals, swamps and other potential breeding sites were also drained. A lack of knowledge in ecology and epidemiology limited the success of these methods. A global initiative to eradicate and control malaria by the WHO succeeded in Europe and Northern America (WHO, 2005). The emergence of drug-resistance and lack of political and financial support prevented the same success rate in poorer countries. Collaborations between different disciplines of science and also with communities are vital for the success of interventions to effectively control malaria.

### 1.2 Global impact of malaria

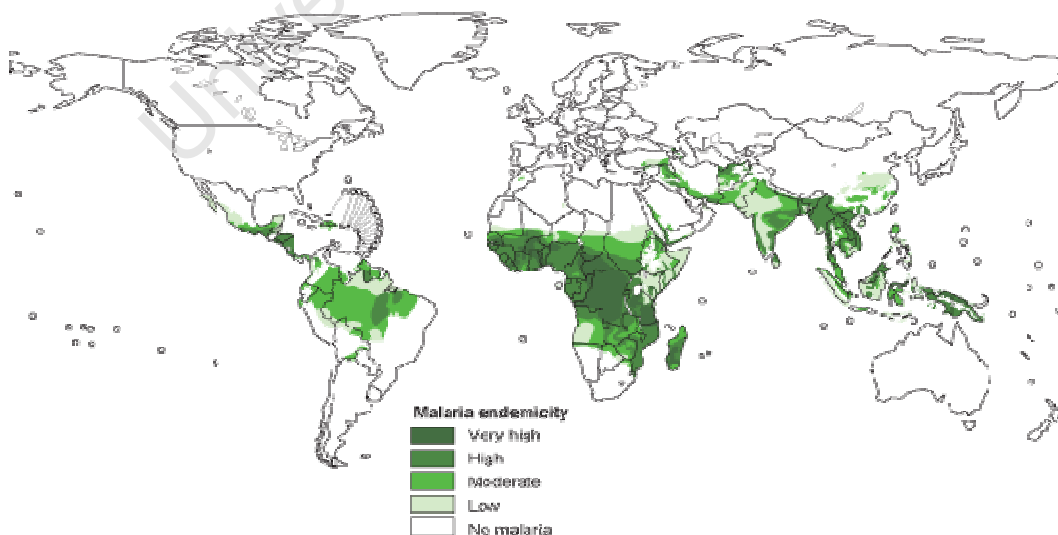


Figure 1 Map of malaria risk countries (WHO, 2005)

Malaria is responsible for more than one million deaths and almost 500 million clinical cases per year (WHO, 2005). Malaria is generally endemic in the tropics extending into the subtropics which include Africa, India, Brazil, Sri-lanka, Thailand, Indonesia, Vietnam, Cambodia, the Middle East and China (WHO, 1999). Approximately 40% of the world's population live in malaria risk areas (Fig 1). More than 80% of malaria deaths occur in sub-Saharan Africa and children under the age of five are the most affected. The World Health Organisation (WHO) estimates that 10 000 women and 200 000 infants die annually as a result of malaria infection during pregnancy (WHO, 2007). People in Africa living in malaria risk areas develop at least one clinical attack per year and according to statistics there is one malaria death every 30 seconds (Greenwood et al., 2002 and Winstanley et al., 2002).

Global warming and travelling have contributed to the spread of malaria. Malaria can be effectively treated but is most prevalent in poorer populations. Nearly 250 times more malaria deaths occur in the poorest countries compared to the richest. Malaria-endemic countries carry great social and economic burdens but more importantly a high cost of human lives. According to the WHO malaria contributes synergistically with HIV/AIDS to morbidity and mortality in areas highly affected by both infections (WHO, 2007). Malaria vector control includes indoor residual spraying of long-acting insecticides and long-lasting insecticidal nets. Antimalarial treatment policies vary between countries and depend on the epidemiology of the disease, transmission, drug resistance and political and economical influence. The staggering statistics associated with this disease has lead to initiatives by global organisations to combat malaria. Despite valiant initiatives by the Global Fund, the Gates Foundation and the WHO the burden of malaria on poor countries is still high (WHO, 2005). The effective prevention and treatment of malaria is estimated at \$3.2 billion per year but only a percentage of that sum is available.

### 1.3 Impact of malaria in South Africa

Malaria risk areas in South Africa include Kwazulu-Natal, Mpumalanga and the Northern Province according to the Malaria in South Africa database (2003). Malaria cases in Kwazulu-Natal have decreased to less than 3 500 for the 2001-2002 malaria season from over 40 000 for the 1999-2000 season. This is thought to be partly a result of re-introducing the spraying of DDT to control the vector, changing the first-line treatment to co-artemether in some areas, and applying a regional approach to malaria control as part of the Lubombo Spatial Development Initiative, which covers eastern Swaziland, southern Mozambique and north-eastern Kwazulu-Natal.

### 1.4 Malaria parasite and vector

A military doctor, Charles Laveran, first discovered the malaria parasite in 1880 and was awarded the Nobel Prize in 1907 for this discovery (CDC, 2004). Malaria is caused by the *Plasmodium* genus of protozoan parasites, namely *Plasmodium falciparum*, *P. vivax*, *P. ovale*, and *P. malariae* (WHO, 2001). The *Plasmodium* species; with the exception of *P. malariae* (may affect the higher primates) are exclusively parasites of man. *P. falciparum* is the most common protozoan in Africa (WHO, 2005). The puzzle of malaria transmission was solved by Ronald Ross in 1897 by identifying the *Anopheline* mosquito as the malaria vector (CDC, 2004). He consequently also received the Nobel Prize in 1902. Only female mosquitoes transmit malaria as they need a blood meal to produce eggs (Moorthy et al., 2004). The life cycle of the parasite is divided between a vertebrate human host and an insect vector. The basic life cycle of the parasite is shown in Figure 2.

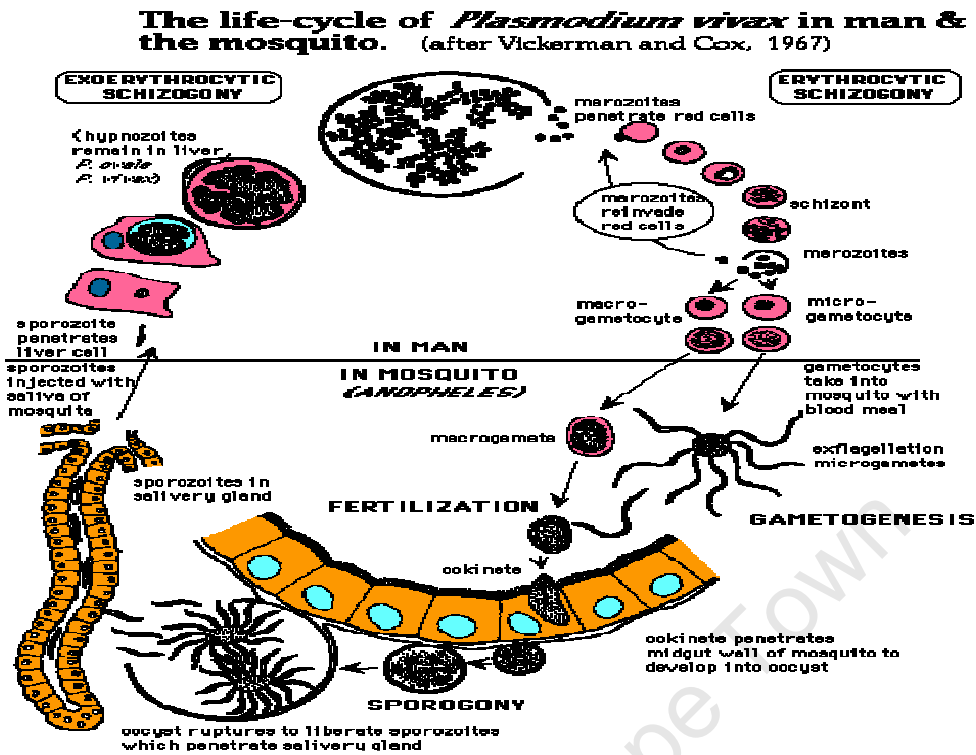


Figure 2 Life cycle of the malaria parasite (WHO, 2001)

Two asexual stages namely exo-erythrocytic and intra-erythrocytic occur in the human host. The exo-erythrocytic stage occurs in the liver and is asymptomatic. If an infected mosquito takes a blood meal from a human it releases a small amount of saliva which contains an anticoagulant and sporozoites into the bloodstream. Once in the bloodstream sporozoites penetrate the liver cells. Sporozoites mature to merozoites which are released from infected liver cells. Merozoites either invade other liver cells or infect erythrocytes. The intra-erythrocytic stage occurs in erythrocytes and is symptomatic. Merozoites enter erythrocytes where it undergoes asexual replication to produce schizonts. Mature schizonts rupture and merozoites are released from infected erythrocytes into the bloodstream. This intra-erythrocytic cycle is repeated every 48 or 72 hours depending on the *Plasmodium* spp. Merozoites can also develop into male or female gametocytes. These gametocytes can be ingested by a mosquito during a blood meal and fuse inside the mid-gut of the vector. Sexual and asexual replication occurs in the mosquito to produce sporozoites. Finally sporozoites

migrate to the salivary glands of the mosquito where they can infect a human host. The main focus of this study will be on the intra-erythrocytic stage of the life cycle.

Several tissues and organs are affected by severe malaria (Miller et al., 2002). The surface of infected red blood cells is modified making it able to adhere to small blood vessels which lead to an obstruction to tissue perfusion. Adhesion of the parasite to endothelium protects it from destruction in the spleen. A principle pathophysiological feature of severe malaria is metabolic acidosis. There are long-term disability risks associated with severe malaria (Winstanley et al., 2002). In pregnancy malaria can cause severe anaemia, low birth weight and an overall low survival rate of the foetus. Cerebral malaria is associated with metabolic acidosis and elevated plasma creatinine levels (Oguche et al., 2002). Other symptoms of malaria include fever, chills, shaking, flu-like symptoms, muscle aches, tiredness, and headache (CDC, 2000). Nausea, vomiting, and diarrhoea may also occur. Malaria may cause anaemia and jaundice because of the loss of red blood cells. An infection with *P. falciparum*, if not promptly treated, may cause kidney failure, seizures, mental confusion, coma and death.

## **1.5 Malaria research**

Research on the biology of the malaria parasite has greatly contributed to the successful development of screening assays and potential vaccines. The development of effective antimalarial treatments has been hindered by the parasite's complex life cycle and extensive polymorphisms in its genome. The malaria parasites use red blood cells to acquire haemoglobin which is used as a source of amino acids needed for parasite growth. Haemoglobin is hydrolysed by protease enzymes in the parasite's food vacuole to form the globin (protein) and heme (Baelmans et al., 2000). Heme is toxic to the parasites and is detoxified or removed (Orjih et al., 1981). During this process the parasite produces a potential toxin, ferriprotoporphyrin. The parasite forms an insoluble polymer hemazoin, also known as the malaria pigment which detoxifies ferriprotoporphyrin. This process is a potential drug target as a compound that inhibits the polymerisation of haematin will be lethal to the parasite. Hemozoin formation may occur spontaneously in the acidic food vacuole of the parasite (Dorn et al., 1995). The structure of hemazoin is identical to  $\beta$ -hematin and an antimalarial

assay to detect compounds inhibiting  $\beta$ -hematin formation was developed (Bohle et al., 1997; Egan et al., 1999 and Ncokazi et al., 2005). The conventional methods for determining parasitemia have been replaced by different types of *in vitro* assays. Early methods for activity testing were mainly using avian malaria models and monkeys (Phillipson et al., 2001). These methods were time-consuming and not suitable for bioassay-guided fractionation. The oldest method of detection was developed by Desjardins et al. (1979) and was based on the incorporation of tritium-labelled hypoxanthine. The lactate dehydrogenase enzyme of *P. falciparum* is essential for energy production of the parasite. The parasite lactate dehydrogenase (pLDH) assay has proven successful as a method to determine parasite viability (Makler et al., 1993 and Basco et al., 1995). A monoclonal antibody-bound immunocapture assay of pLDH has been developed to combat the influence of colour with crude herbal extracts in the standard pLDH assay (Xu et al., 2007). DNA-based antimalarial assays using fluorescence dye has also been developed (Smeijsters et al., 1996).

The recent sequencing of the genomes of *P. falciparum* and *A. gambiae* has cleared the way for developing new drug and vaccine targets (de Roode et al., 2003). Advances in technology have assisted in the creation of transgenic mosquitoes which do not transmit malaria (Ito et al., 2002). This has lead to concerns about the effect of these transgenic mosquitoes on humans and the environment as well as the possible evolution of the parasite. Attempts to develop a vaccine have been hindered by the parasite's complex life cycle. Protozoa, unlike pathogenic bacteria, share many biochemical pathways with their human host. Early attempts at vaccination included literally being bitten by about a thousand malaria-infected mosquitoes that had been X irradiated. The genomes of *P. falciparum* and *A. gambiae* have identified approximately 5300 *P. falciparum* antigens. Genetic vaccination with plasmid-DNA expression encodes for a relevant antigen without using live agents (Martin et al., 1999). DNA vaccines have proven to be safe, well-tolerated and immunogenic (Parker et al., 1999 and Wang et al., 1998). A vaccine based on plasmid-DNA has advanced to clinical trials (Martin et al., 1999). Vaccine development against malaria is still under investigation. With all this information and technologies in hand, the possibilities for developing a successful vaccine still elude molecular scientists.



## 1.6 Background of traditional medicine

Ancient civilisation recorded the use of traditional medicine for the treatment of malaria and more than 1 000 plant species are used to treat fevers and malaria (WHO, 1986 and Willcox et al., 2001). Traditional medicine is cheap and easily available in developing countries. Traditional healers are more common in developing countries than medical doctors. Religion and culture are important as the use of a medicinal plant usually accompanies some sort of ritual. Parasite-resistance to whole-plant extracts has not been reported which might be due to the synergistic effect of various compounds within a whole-plant extract (Phillipson et al., 1991 and Willcox et al., 2000). Phytotherapy may have fewer side effects due to many active compounds working together and administered at smaller doses. Whole-plant extracts are still used as an alternative antimalarial treatment especially in developing countries. Two of the most potent antimalarial agents in history namely quinine and artemisinin were isolated from medicinal plants. It is estimated that 50% of prescription drugs originate from natural products (Edinburg et al., 1998).

Most traditional medicine (TM) is given orally but some plant extracts are given rectally, topically applied or inhaled (Edinburg et al., 1998). Although parenteral administration is not widely used, some medicinal plants are placed in a small incision in the skin to enhance the effect. Several factors influence the determination of a dose, such as size, age, constitution and severity of the disease. Measurements are often approximate in the absence of scales or measuring containers. Most traditional medicines are freshly prepared. Lipophilic compounds like artemisinin can be administered at lower doses in an aqueous infusion (Phillipson 1991). With aqueous preparations lower toxicity and synergism of different compounds can be observed. Studies have shown the potential harm of combining standard antimalarials with TM (Ekong et al., 1991 and Kirby et al., 1995a). Combinations can lead to sub-therapeutic exposure of the drug to the parasite which can aid in the development of drug-resistance. However not all combinations of chloroquine and TM's are antagonistic (Muregi et al., 2003). Some medicinal plants can exert activity which might not be primarily antiprotozoal but might address the symptoms associated with malaria. Many populations living in malaria-risk areas acquire natural immunity. Medicinal plants which alleviate symptoms and reduce parasitemia can assist in natural

immunity (Kirby et al., 1996). Health care systems in most developing countries are not satisfactory. Poverty, lack of expertise and political instability further increase the dependence of communities on cheap and accessible indigenous medicine. The more primitive plants are used as foods while the more evolved species are used as medicine (Hedberg et al., 1993). There are more plants reported for medicinal use than reported as foods. Most plants have evolved secondary metabolites as a defence mechanism against mammals and insects. The Food and Drug Association has classified herbal substances as dietary supplements under the Dietary Supplement and Health Act of 1994 (Marcus and Grollman et al., 2002). This has contributed to a lack of control in this field.

A single active constituent cannot always replace the whole plant extract. *A. annua* contains a plethora of compounds including artemisinin (Phillipson et al., 2001). The blood red sap from the South American *Croton* sp. is traditionally used to treat wounds and is commonly known as 'Dragons blood'. This natural product has been investigated for its traditional uses and the constituents were found to work synergistically in wound healing. Clinical studies with a herbal mixture of ten plants showed that all ten herbs are needed for clinical effectiveness. A multi-disciplinary approach is needed to honour the full potential of TM. The majority of populations in developing countries rely on TM. Science has evolved and continues to build on current technologies. The clinical evaluation of TM for quality, safety and efficacy can be achieved using acceptable protocols. To date we have only dented the surface of biodiversity as the vast majority of the world's medicinal plants have not been investigated for biological activity. Tropical rain-forests are under threat and with it the annihilation of hundreds of plant species (Phillipson et al., 1994). An estimated 25% of all plant species will be extinct by the mid-21<sup>st</sup> century. The scientific investigation of our natural resources might lead to the discovery of novel antiplasmodial compounds.

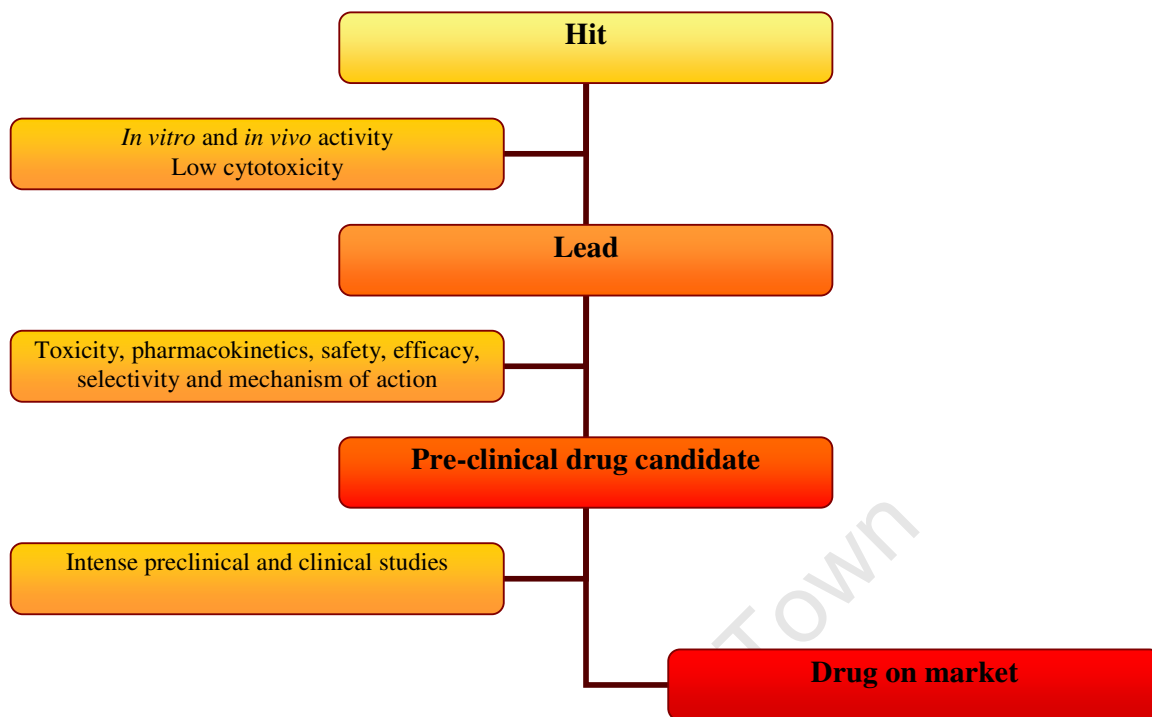
## **1.7 Traditional medicine in South Africa**

The indigenous knowledge of South Africa date back to 1 000 BC which is long before Europeans discovered the southern tip of Africa as a gateway to the East

(Edinburg et al., 1998). Southern Africa has more than 30 000 species of higher plants which makes it a biodiversity hot spot (Van Wyk et al., 1997). The Cape Floral Kingdom alone has almost 9 000 plant species making this region the most diverse temperate floral kingdom on earth, rivalling the tropical rainforests in terms of species richness. South Africa has a remarkable biodiversity and cultural diversity with approximately 3 000 species of medicinal plants. There are approximately 200 000 traditional healers in South Africa, and more than 50% of the South African population consult these healers, usually in addition to using modern biomedical services. Nearly 400 species of indigenous plants are sold commercially as TM in Kwazulu-Natal, South Africa (Kubukeli et al., 1999). The value of an ethnobotanical approach to drug discovery was demonstrated with antiplasmodial studies on South African medicinal plants which yielded several promising plant extracts (Prozesky et al., 2001 and Clarkson et al., 2004). Traditional healers can be potential partners in the delivery of primary health care. The WHO defines health as a complete state of physical, social and mental well-being and the practice of traditional healers who examine the whole being (physical, mental and spiritual) is in line with this definition. African traditional healing practices vary between countries depending on social and cultural heritage and traditions. Although South Africa has such a rich plant resource, very little antimalarial work has been done on South African plants. This is probably due to the lack of malaria incidences in South Africa which makes the search for medicinal plants used as antimalarial treatment difficult.

## **1.8 Drug discovery and development**

Conventional drug development takes at least ten years and the cost associated with drug discovery and development is estimated at \$800 million per drug entity reaching the market (Shearer et al., 2005). Several steps are involved in getting a potential drug from a test tube to human subjects. The common processes in drug discovery are illustrated in Scheme 1.



Scheme 1 Drug discovery process

During the 1975-1996 period, 1 223 new drugs were developed and only three were antimalarial drugs. *Plasmodium* is one of the most researched causative organisms especially in the field of plant medicine (Phillipson et al., 2001). Antimalarial compounds must be selective towards the parasite without harming the human host. Plants have an advantage from millennia of evolution to create a rich source of chemically diverse compounds. Ironically the chemical diversity and novelty of natural products is also a disadvantage as supply and access often discourage research sponsored by pharmaceutical companies (Phillipson et al., 1991). Chemical synthesis of natural products can be complex and often economically unviable. Pharmaceutical companies are looking towards combinatorial chemistry and high throughput screening for drug development (Eddershaw et al., 1999). However the development of large libraries of compounds has not delivered the expected results (Hedberg et al., 1993). Lipinski's 'Rule of 5' serves as a drug-design guideline which can aid in absorption (Lipinski et al., 1997). This rule limits the design of compounds but its usefulness in predicting well-absorbed molecules is noteworthy. Natural products have produced effective antimalarial compounds, which also serve as a template for

developing synthetic and semisynthetic compounds (Mueller et al., 2000). Structure activity relationships have contributed significantly in antimalarial drug development (Tagboto et al., 2001).

The discovery of the antimalarial drug artemisinin by the Chinese focused the attention of antimalarial drug development back to medicinal plants (Mueller et al., 2000). Currently there are no effective alternatives to artemisinin in the treatment of *P. falciparum* malaria on the market or in the late stages of drug development (WHO, 2007). Tang et al. (2006) encourage an efficacy driven approach to investigate traditional Chinese medicine. This evidence-based approach proposes the study of TM already in use. Throughout history medical interventions such as penicillin and the smallpox vaccine were implemented without the knowledge of mechanism of action. Clinical evaluation of TM requires a combination of methodologies. The outcome or benefits of a clinical trial must be clearly defined. The investigation of herbal formulations such as Changshan (*Dichroa febrifua*) showed that a single active approach to drug discovery is not always viable (Willcox et al., 2004a). Changshan studies highlight that false negatives, using this approach with regard to TM research, can occur as some plants only work in combination. Several success stories of a ethnobotanical or phytochemical approach to drug discovery include morphine from the opium poppy, tubocurarine (*Chondrodendron tomentosum*), vinblastine and vincristine (*Catharanthus roseas*) and taxol (*Taxus brevifolia*) (Phillipson et al., 1994). These compounds among several other natural products serve as template molecules for synthetic analogues. Advances in plant biotechnology have improved the understanding of biosynthetic pathways and enzymes involved in the production of active constituents. Plant cell cultivation has been successfully used to produce higher concentrations of active components (Choi et al., 1999). Substances of pharmaceutical interest such as taxol are produced using biotechnological processes involving the cultivation of bacteria, yeast, plant or mammalian cells. The ethnobotanical approach to drug discovery is less expensive in terms of technology than the highly technological approaches (Kirby et al., 1996).

The process of TM investigation must take into account the intended route of administration as well as the traditional route of administration. Pharmacokinetic

evaluation of lead compounds includes absorption, distribution, metabolism and excretion (ADME) (Shearer et al., 2005). Drug metabolising enzymes play an important role in the development of lead compounds. Virtual ADME studies can be conducted without putting on a laboratory coat. Drug interactions are clinically important and are influenced by drug-, patient- and administration-related factors (Dresser et al., 2000). Interactions leading to higher plasma concentrations can enhance adverse effects and / or efficacy. The CYP enzymes are responsible for phase I hepatic metabolism of a broad spectrum of therapeutic drugs. The system for metabolising compounds and the means of analysing the metabolic reaction is very important. The ideal is to mimic exactly the *in vivo* situation through comprehensive *in vitro* screens. Cytochrome P450 (CYP) enzymes and human and liver microsomes are routinely used for *in vitro* metabolism studies. *In vitro* screens unlike *in vivo* screens require less material, they are cost effective and there are no ethical controversies. *In vitro* systems using Caco-2 cell-lines have been developed to predict possible drug absorption by the small intestine epithelium (Hilgers et al., 1990). CYP 3A4 is one of the most important human enzymes responsible for the biotransformation of 60% of oxidised drugs (Dresser et al., 2000). Novel methods for *in vivo* evaluation of specific enzymes such as CYP 3A4 have been developed (Granvil et al., 2003). Pharmacokinetic properties of a potential drug should be evaluated early during preclinical drug development. Although great progress has been made in the field of *in vitro* metabolism it still cannot simulate the complexity of an *in vivo* system. There is a need to develop rapid, cost-effective and 'animal friendly' methods to evaluate *in vivo* metabolism.

Advances in analytical techniques have revolutionised medicinal plant research (Phillipson et al., 2003). Powerful techniques such as high performance liquid chromatography, mass spectrometry and nuclear magnetic resonance (NMR) have had a phenomenal effect on phytochemical research. Hyphenated bioanalytical techniques such as NMR-solid phase extraction have enabled rapid identification of known and unknown compounds irrespective of their concentration in medicinal plants (Clarkson et al., 2006). Despite all these advances in technology the majority of plants are yet to be explored. The use of traditional medicine will continue in the developing world.

Therefore the scientific investigation of traditional medicine to prove efficacy and safety will be of great benefit to these communities.

## **1.9 Natural products and antimalarial chemotherapy**

*Plasmodium* has developed resistance to most antimalarial drugs and the mosquito vector has developed insecticide-resistance (Moorthy et al., 2004). Haemoglobin breakdown is necessary for the action of chloroquine (CQ) and artemisinin (Peters et al., 1986). Understanding the mechanism of action of a drug aids in understanding the development of resistance to the drug (Hyde et al., 2002).

### **1.9.1 *Cinchona***

#### **1.9.1.1 History of *Cinchona***

The *Cinchona* (Rubiaceae) bark discovered in Peru in the early 17<sup>th</sup> century is known as one of the oldest remedies for malaria (Willcox et al., 2004a). This genus is indigenous to South America. People with different backgrounds from royalty to common workers used the plant throughout history but its discovery is still debated today. Great secrecy surrounded this traditional medicine which resulted in the loss of information concerning its traditional preparation. The most important *Cinchona* spp. for pharmaceutical and commercial purposes are *C. ledgeriana*, *C. succirubra* and *C. officinalis*.

#### **1.9.1.2 Quinine-based antimalarials**

Two alkaloids cinchonine and quinine were isolated from the *Cinchona* bark in the early 19<sup>th</sup> century. This was a major breakthrough and later resulted in the formulation of synthetic antimalarials such as amodiaquine, CQ and mefloquine which is based on quinine. A further 30 alkaloids were isolated from the bark but the most potent *in vitro* compounds were quinidine, dihydroquinidine and cinchonine (Karle et al., 1999).

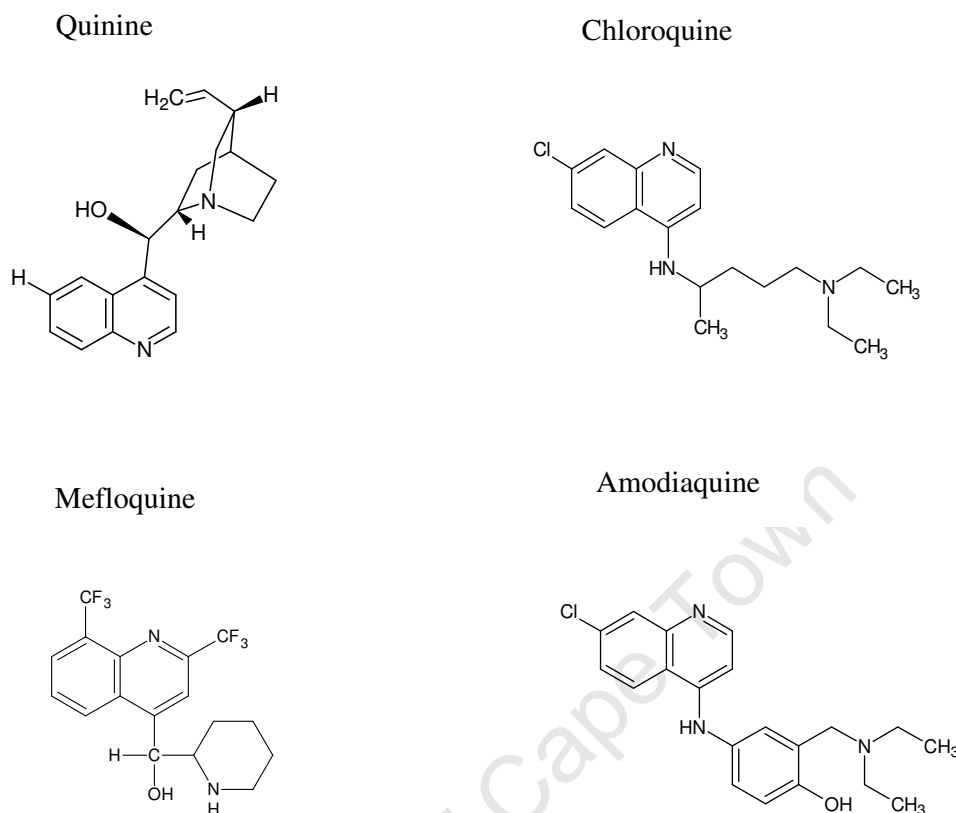


Figure 3 Chemical structures of the quinoline-based antimalarials

Quinine was discovered in a time when the world was in desperate need for antimalarial drugs and the fact that these compounds originated from traditional medicine already in use outweighed the risk of no treatment. Several clinical trials on *Cinchona* and its alkaloids were conducted in the early 20<sup>th</sup> century (Waters et al., 1916; Acton et al., 1920 and Hicks et al., 1935). The pharmacokinetics and mechanism of action of *Cinchona* alkaloids were studied decades after it was first clinically used. The *Cinchona* bark and its alkaloids especially quinine were extensively investigated. Studies suggested that the crude extract is as effective as quinine. Quinine and quinidine have good oral bioavailability (White et al., 1996). Side-effects associated with *Cinchona* alkaloids include hearing impairment, nausea and visual disturbances (Karbwang et al., 1993 and White et al., 1981). Fletcher et al. (1934) also stated that the “bitter taste is enough to make healthy people vomit, let alone those ill with malaria”.



The difficulties encountered with antimalarial vaccines have resulted in scientists re-evaluating current antimalarial drugs such as CQ. Cerebral malaria is associated with over-stimulation of the immune system and cytokine network in particular tumour necrosis factor (TNF) and interleukin-6 (IL-6) (Kwiatkowski et al., 1990; Picot et al., 1990 and Aderka et al., 1989). Picot and co-workers (1993) showed that CQ inhibits the secretion of TNF and IL-6. TNF inhibition was caused by disrupting normal iron metabolism. The inhibition of cytokine secretion in the pathophysiology of cerebral malaria can enhance patient survival without having a direct effect on the parasitemia. CQ might be useful as a prophylactic agent in cerebral malaria.

Synergism observed with different components might be an effective treatment against the quinine-resistant parasite. A recent study showed that the combination of chloroquine and certain plant extracts leads to a synergistic effect against multi-drug resistant *P. falciparum* (Muregi et al., 2003). The combination of quinine with quinidine and cinchonine was more effective against the quinine-resistant parasites and even a mixture of alkaloids was more effective than a single compound (Druihle et al., 1988). A combination of alkaloids was as effective as quinine and fewer side-effects were also observed (Bunnag et al., 1989). In this modern age the *Cinchona* bark is still being used to treat malaria in several countries. The legacy of *Cinchona* specifically its contribution to antimalarials has greatly benefited the human race.

### **1.9.1.3 Mechanism of action and resistance**

This class of antimalarial drugs act by binding to free heme thus preventing the conversion to non-toxic hemazoin. Quinoline antimalarials accumulate in the acidic food vacuole of the parasite which is partly due to its basic nature. CQ accumulation is much less in resistant strains than in sensitive strains of the parasite. Early studies showed that the efflux of CQ is 40-50 times more with chloroquine-resistant (CQR) strains than chloroquine-sensitive (CQS) strains of *P. falciparum* (Krogstad et al., 1987). Resistance might be due to a decrease in the influx of CQ or an increase in the efflux of CQ or a reduction in the binding affinity of the drug to its target molecule. These hypotheses have been extensively investigated over the years. CQ was recommended as the first-line treatment for malaria by the WHO for decades (Willcox

et al., 2004b). Increasing levels of drug-resistance has resulted in a change of first-line treatment for malaria. Mefloquine (MQ) has been used to treat CQR malaria. Unfortunately resistance to MQ has evolved over recent years. P-glycoprotein (P-gp) is an ATP-binding molecule located on the plasma membrane of the cell and has been linked to the development of resistance in cancer (Grossi et al., 2004). A P-gp homologue (Pgh-1) is located on the food vacuole of *P. falciparum* (Cowman et al., 1991). *P. falciparum* multidrug-resistant (pfmdr-1) gene is known as the gene encoding Pgh-1 and is amplified in CQR strains of *P. falciparum*. Additionally mutations in Pgh-1 have been associated with CQR parasites (Reed et al., 2000). Pgh-1 can serve as an efflux pump which may lead to reduced drug concentrations in the food vacuole. MQ-resistance is also associated with an increase in pfmdr-1 gene expression (Price et al., 2004). The antidepressant fluoxetine is a P-gp inhibitor and has shown to enhance the susceptibility of resistant *P. falciparum* to CQ (Coutaux et al., 1994 and Singh et al., 2000). Further studies showed that fluoxetine potentiates the effect of CQ and MQ on multidrug-resistant *P. falciparum* (Khairul et al., 2006).

Antihistaminic compounds have demonstrated CQ resistance reversal properties against *in vitro* and *in vivo* strains of *Plasmodium* (Peters et al., 1990). Several studies demonstrated the resistance reversal effect of a calcium channel blocker verapamil (Bray et al., 1994 and Martin et al., 1987). It was commonly accepted that an increase in the pH of the food vacuole might account for the decreased accumulation of CQ inside the food vacuole thus leading to resistance. However studies by Ursos and co-workers (2000) contradicted this hypothesis and showed a decrease in the pH of the food vacuole. Further studies showed that the pH of the food vacuole influences the ratio of soluble/insoluble heme (Dzekunov et al., 2000). The lower pH promotes the formation of insoluble heme thus reduces the amount of soluble heme available for CQ binding. The exact mechanism of resistance is still debated and it is possible that CQ-resistance is due to a combination of mechanisms.

## **1.9.2 *Artemisia annua***

### **1.9.2.1 History of *Artemisia annua***

*Artemisia annua* is indigenous to China and references in ancient Chinese documents date back as far as 168 BC (Hien et al., 1993). The ancient Chinese referred to this plant as ‘Qing Hao’ which means ‘green herb’. The Chinese have used *A. annua* for at least 2000 years to treat fevers and malaria (Meshnick et al., 2002). This plant was traditionally administered as a tea. Antimalarial activity was observed with other *Artemisia* sp. other than *A. annua* (Valencha et al., 1994). These other species of *Artemisia* most likely contain novel antimalarial compounds. Clinical efficacy of *A. annua* has not been well studied. Several clinical studies showed that herbal preparations of *A. annua* are effective against malaria (Hirt et al., 2000 and Mueller et al., 2000). The use of herbal preparations of this plant could be an alternative treatment for the large number of patients who do not access treatment due to a lack of antimalarials. Small-scale cultivation and local utilisation of the herbal preparation of *A. annua* is encouraged by non-governmental organisations (Anamed, 2008). However aqueous preparations of *A. annua* contain significantly lower artemisinin (ART) concentrations than the WHO recommendations (Räth et al., 2004). These low concentrations in herbal remedies can lead to the development of resistance.

### **1.9.2.2 Artemisinin-based antimalarials**

ART was first isolated in China in 1971 from *A. annua* (Hien et al., 1993). The leaves and flowers of the plant contain ART. Tan et al. (1998) showed that only two other *Artemisia* spp. namely *A. apiacea* and *A. lancea* contain ART. The semisynthetic derivatives artesunate, artemether and arteether have been developed from ART (Mueller et al., 2000).

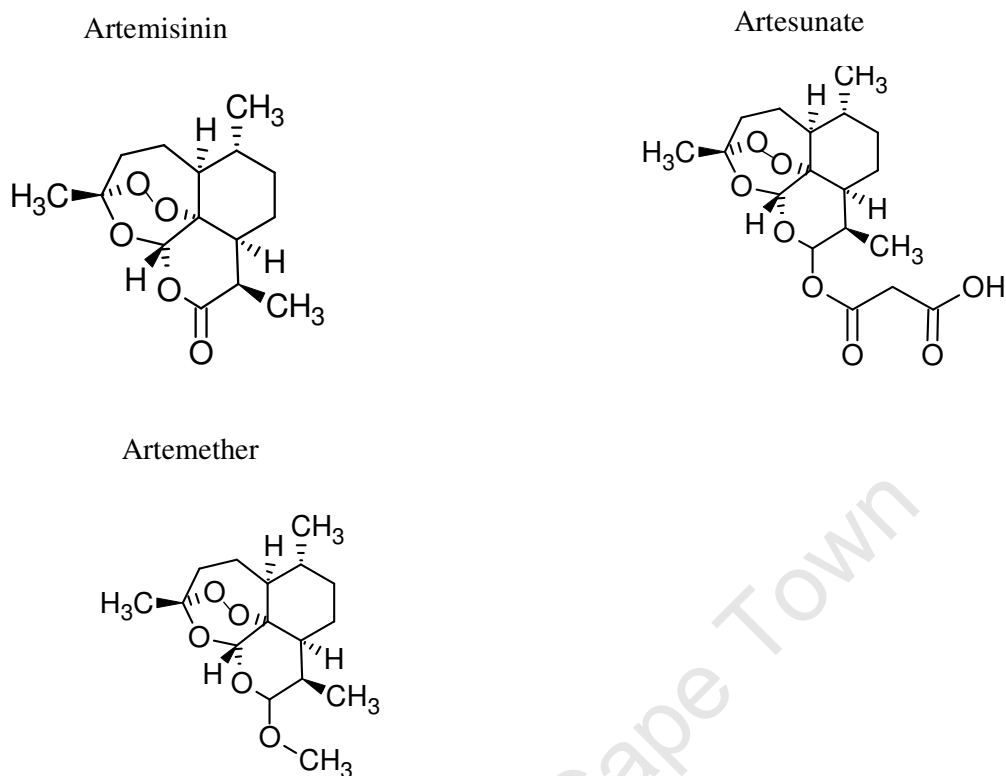


Figure 4 Chemical structures of artemisinin and its derivatives

ART and its derivatives are the most powerful antimalarial drugs in history and act as prodrugs that have the same active metabolite, dihydroartemisinin (White et al., 1994). There are no reports on the toxic effects of *A. annua* or ART, although at high doses ART can be neurotoxic but this has not been observed in clinical studies. The administration of ART is limited to oral and rectal routes due to the poor solubility of ART in oil and water (Ashton et al., 1998). Synthetic analogues artesunate and artemether of ART are more soluble and can be administered intravenously and intramuscularly (Van Agtmael et al 1999). Artesunate has the most rapid therapeutic response (White et al., 1994). ART is a sesquiterpene lactone containing an endoperoxide group which bridges an oxygen-containing ring. The structure of ART has challenged the minds of many medicinal chemists but no cost-effective synthesis has been developed. The aerial parts of *A. annua* are still the major source of ART. Biotechnology has enabled the commercial cultivation of *A. annua* to maximise the yields of the active component (Laughlin et al., 2002).

ART is one of twenty nine sesquiterpenes in *A. annua* and many of these compounds are in greater quantities than ART in wild strains of the plant (Liu et al., 1992). ART is a lipophilic compound but the traditional preparation of *A. annua* is as an aqueous decoction. Congener constituents of the plant can aid in solubility and enhance the activity of ART as demonstrated *in vitro* with flavonoids. Further studies reported that flavones have no effect on heme but catalyse the reaction of ART with heme (Bilia et al., 2002). Flavones can also help with the solubility of ART in the herbal preparation. Several studies concluded that the antimalarial properties of *A. annua* are not only due to ART but a combination of compounds. Different compounds in the herbal preparation might inhibit metabolising enzymes. The pharmacokinetics of ART from the herbal preparation has not been fully studied. Recent studies by R  th et al. (2004) showed that the bioavailability of ART from an infusion of *A. annua* is considerably less than pure ART. Treatment with ART results in a decrease of ART concentration in plasma and an increased concentration of the metabolite dehydroartemisinin (Van Agtmael et al., 1999). Contrary to modern belief combination therapy has been used in traditional medicine for hundreds of years.

ART unlike quinine-based and antifolate antimalarials is gametocytocidal thus reducing the potential transmission of malaria (Price et al., 1996). The commonly quoted affordable limit of \$1 for a course of antimalarial treatment was already too high for developing countries (Wilcox et al., 2001 and Wilcox et al., 2004a). The WHO recommends that these drugs be used in combination with other antimalarial drugs. The cost of ART-based combination therapy is effective yet unaffordable to the communities which need it the most. This sensitive situation has led to exploitation as fake artesunate is being sold in Southeast Asia (Newton et al., 2002).

### **1.9.2.3 Mechanism of action and resistance**

The structure of ART is very different from quinine-derived antimalarials. Quinine alkaloids are basic and ART is neutral and non-polar. Given the structural difference between these two classes of drugs the mechanism of action against the malaria parasite is also different. The mechanism of action of ART involves its interaction with heme and the newly formed free radicals derived from ART further react with

parasite molecules thus disrupting the normal metabolic pathways of the parasite. The mechanism of action of ART still remains a controversial subject. Several hypotheses exist which include its reaction with heme and the reduction of the peroxide by ferroprotophyrin IX resulting in active intermediates which can react with heme and essential parasite proteins. Ponwee and co-workers (2007) showed that ART interacts with heme and heme-containing proteins which results in reduced ART effectiveness. The exact method by which ART-reactive products interfere with metabolic pathways and kill the parasite is still not clear (Orjih et al., 1996). The activity of ART is specific as it reacts only with free heme and not with haemoglobin of uninfected red blood cells (Hong et al., 1994). The effect of ART is antagonised by iron chelators and also by CQ which binds to heme (Meshnick et al., 1993). The mechanism of action of ART is also associated with the alkylation of heme and the parasite mitochondria and membrane (Maeno et al., 1993 and Meshnick et al., 1994). ART and its derivatives without combination therapy can result in recrudescence after two weeks of treatment which might be due to insufficient availability of heme as the parasites might not be in the growth phase during exposure to the drug. Furthermore the WHO has banned the use of oral ART monotherapies in an effort to prevent the development of drug-resistance (WHO, 2007).

An artemether-quinine meta-analysis of clinical trials showed that artemether is at least as effective as quinine and resulted in fewer serious adverse effects. Chinese *Plasmodium* strains are less sensitive to ART than African strains (Wongsrichanalai et al., 1997). This might be due to the centuries of exposure to *A. annua* in China. Resistance is more likely to develop against a single compound than a combination of compounds as found in herbal preparations. ART-resistant parasites have been cultivated *in vitro* with  $\alpha$ -thalassemic erythrocytes (Ponmee et al., 2007). The shorter half-life of ART can be advantageous against resistance development because the parasite is exposed to shorter periods of sub-therapeutic levels. ART may stimulate the immune system in response to malaria (Ye et al., 1982).

### 1.9.3 Antifolate antimalarial drugs

Antifolates are the oldest antimetabolite class of anticancer drugs. CQ-resistance led to antifolate treatment of malaria mainly using combinations of pyrimethamine (PYR) and sulfadoxine (SFD). Antifolate chemotherapy is widely used for malaria however their continued use has been hindered by the emergence of resistance (Plowe et al., 1998).

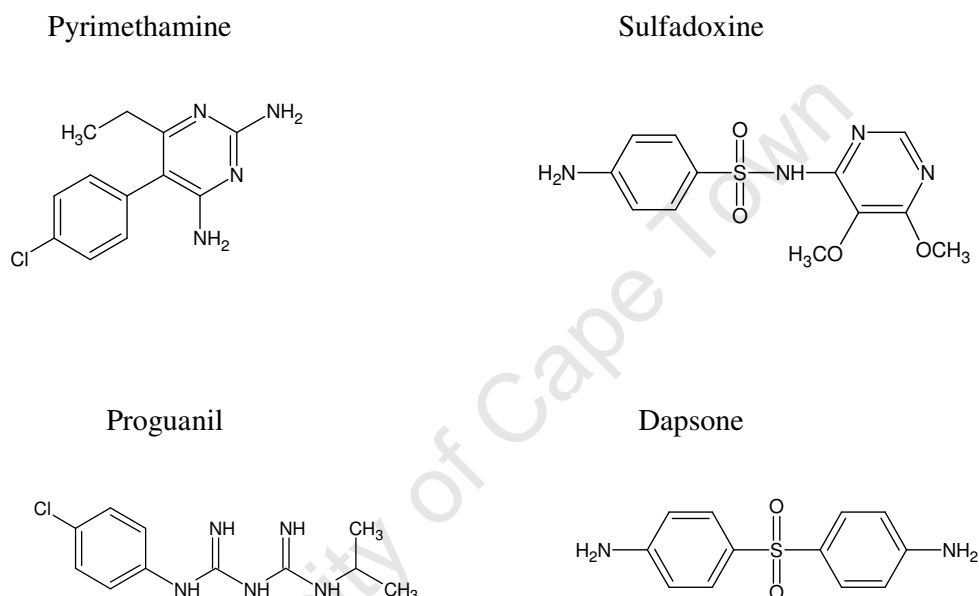


Figure 5 Chemical structures of the antifolate drugs

The molecular targets of these drugs are folate-dependent enzymes defined as dihydrofolate reductase (dhfr) for PYR and dihydropteroate synthetase (dhps) for SFD (Wang et al., 1997). Mutations in the dhfr and dhps genes are known causes of resistance.

### 1.9.4 Promising antimalarial medicinal plants and plant-based compounds

The antimalarial activity of approximately 600 plants representing 129 families was published by Spencer et al. (1947). Several plants were identified as promising leads including the Asian medicinal plant *Brucea javanica*. Antiplasmodial quassinoids were later isolated from the fruits of *B. javanica* (O'Neill et al., 1987). *In vivo* studies on the crude extract showed high activity but also high toxicity. The observed toxicity lends some support in the aqueous preparation used traditionally. Allen and co-workers (1994) showed that the aqueous extract of *B. javanica* antagonised the effect of CQ *in vitro* against CQR strains of *P. falciparum*. These studies suggested that co-administration of this TM with CQ might lead to treatment failure which can subsequently result in the development of resistance.

Berberine occurs in several plant families including Annonaceae and Menispermaceae which is widely used in the treatment of malaria, amoebiasis and leishmaniasis (Phillipson et al., 1993 and Vennerstrom et al., 1988). This alkaloid has shown to be active *in vitro* against *P. falciparum* but no *in vivo* activity was observed. *Cryptolepis sanguinolenta* is traditionally used in Ghana to treat malaria and was reported to be clinically effective (Kirby et al., 1995b and Paulo et al., 2000). An antiplasmodial indoloquinoline, cryptolepine, was isolated from this plant. Cryptolepine, like berberine, was active *in vitro* against *P. falciparum* but did not show *in vivo* activity. Studies suggested that cryptolepine intercalate with DNA which could account for its potent *in vitro* activity.

Changshan is an ancient Chinese febrifuge mixture of plants with the key ingredient being *Dichroa febrifuga* (Miyasita et al., 1979). The active constituents of *D. febrifuga* namely  $\beta$ -dichroine (febrifugine) and  $\alpha$ -dichroine (isofebrifugine) were isolated early in the twentieth century (Jang et al., 1948 and Koepfli et al., 1950). The antiplasmodial activity of *D. febrifuga* was observed against *in vivo* avian malaria (Spencer et al., 1947). Febrifugine was 100 fold more active than quinine in an avian malaria model (Koepfli et al., 1947). Ultimately the emetic effect of *D. febrifuga* and its alkaloids hindered the development of these as antimalarial drugs.



Phaeanthine is a bisbenzylisoquinoline alkaloid isolated from *Triclisia patens* was shown to be more active *in vitro* against the CQR K1 strain than the CQS T9-96 strain of *P. falciparum* (Ekong et al., 1991). Combination experiments with phaeanthine and CQ showed antagonism against the CQS strain and an additive effect against the CQR strain.

The 1,2,4-trioxane group of the ART molecule produces radicals which cause parasite death (Robert et al., 2002). Chimeric compounds named trioxaquinines contain the trioxane entity responsible for ART activity and an aminoquinoline entity responsible for antiparasmodial potency of CQ (Basco et al., 2001). Trioxaquinines have shown promising *in vitro* and *in vivo* activity against CQR strains (Dechy-Cabaret et al., 2004 and Benoit-Vical et al., 2007). This class of compounds is active against both sexual and asexual stages of the parasite life-cycle and also showed low cytotoxicity. These drugs unlike ART can be chemically synthesised which would make them more affordable.

### 1.10 Scope of the study

The purpose of this study was to use an ethnopharmacological approach to isolate novel antiplasmodial compounds from *Siphonochilus aethiopicus* and *Aloe ferox*.

The first objective was to isolate and characterise novel antiplasmodial compounds and evaluate schizontocidal activity using a malaria-mouse model.

The processes followed were:

- *In vitro* and *in vivo* bioactivity testing as well as *in vitro* cytotoxicity testing
- Isolation and structural elucidation of antiplasmodial compounds

Secondly, the objective was to investigate the bioavailability and metabolism of the most promising compound in an *in vivo* system.

The processes followed were:

- Bioavailability study of the most active compound
- Metabolism investigation

# *Chapter 2*

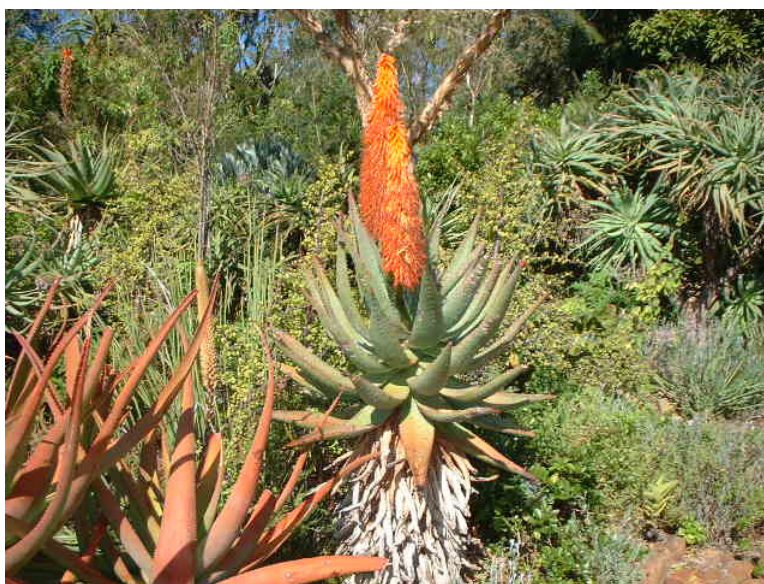
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## *Literature review of plants*

## 2 Literature review of plants

*Aloe ferox* and *Siphonochilus aethiopicus* are commonly used as traditional medicines. These two medicinal plants were selected for this study based on traditional knowledge and previous antiparasmodial activity.

### 2.1 *Aloe ferox*



*Aloe ferox* photographed by C.Lategan (Kirstenbosch Botanical Gardens, Cape Town)

*Aloe ferox* Mill. Asphodelaceae (formerly classified as Liliaceae) is a single stemmed *Aloe* which produces red or orange flowers from May to August (van Wyk et al., 1996 and Hutchings et al., 1996). *Ferox* means ferocious which refers to the prickly leaves. There are approximately 330 *Aloe* species (Trease and Evans et al., 1978). The history of aloes dates back to the fourth century B.C. and was well known by the Greeks. The aloe plant is believed to be the main reason for Alexander the Great to conquer the island of Socotra. The mystery that surrounds aloes still intrigues researchers today.



*A. ferox* inner leaf gel and outer leaf photographed by C.Lategan



Dried leaf exudates of *A. ferox* (Cape Aloe) and products of Cape aloe. Photographs obtained from Medicinal Plants of South Africa (van Wyk et al., 1997)

*Aloes* are used all over the world by various populations and the species used depends mainly on availability in local regions (Loots et al., 2007). *A. ferox* is indigenous to South Africa and is widely distributed from Swellendam to the Western Cape, Eastern Cape and southern Kwazulu-Natal provinces, some parts of south-western Lesotho and south-eastern Free State regions (van Wyk et al., 1996). *A. ferox* is commonly known as bitteraalwyn (Afrikaans), bitter aloe (English) or umhlaba (Xhosa, Zulu or Sotho) (van Wyk et al., 1997). *A. ferox* has extensive medicinal usage ranging from an insect repellent, the healing of sores, blisters, cuts; treating acne, burns, sinusitis, syphilis, stomach complaints and various other ailments (TRAMED III, 2001). The leaves or roots are boiled in water to treat arthritis, eczema, conjunctivitis, hypertension, stress and are also used as a laxative. Fresh leaf juice is used to treat ophthalmia and ground leaves are used as snuff (Hutchings et al., 1996). Venereal sores are treated with leaf decoctions and powder of charred ground leaves. The nectar might have a narcotic effect (Watt and Breyer-Brandwijk et al., 1962). A medicinal product derived from *A. ferox* called Cape Aloe is used for stomach complaints and as a laxative. This product was first exported in 1780 and is now mainly exported to Europe but also marketed and used locally (Trease and Evans et al., 1978; van Wyk et al., 1996). Cape Aloe is still prepared by an age old method with little modification. Aloin is known as the active compound responsible for the purgative characteristic of *A. ferox*. It was found that aloin acts as a prodrug for aloe-emodin which is formed in the colon. A guideline for export quality Cape Aloe product is that it should contain at least 18% aloin. Seasonal variation of aloin in leaf juice has been observed (Trease and Evans et al., 1996). Aloe gel mainly from *A. vera* is widely used in the food and cosmetic industry (van Wyk et al., 1996). *Aloe* plants are cultivated for commercial purposes in the West Indies, East Africa, north-west India and southern China. *A. ferox* leaf gel is now also being produced for these lucrative industries. The gel which is rich in polysaccharides, polyphenols, phytosterols and indoles may be of therapeutic benefit (Loots et al., 2007).

*A. ferox* is used to treat sexually transmitted infections in the Eastern Cape region of South Africa and in the treatment of syphilis in West Africa (Kambizi et al., 2004, Watt and Breyer-Brandwijk et al., 1962 and Hutchings et al., 1996). The investigation of this plant for its antimicrobial activity led to the isolation of three known

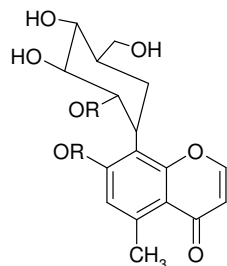
compounds namely aloe-emodin, chrysophanol and aloin A. Aloe-emodin has been reported to have anti-tumor activity (Trease and Evans et al., 1978). Metabolism studies revealed that chrysophanol undergoes biotransformation by CYP enzymes to produce aloe-emodin (Mueller et al., 1998). Genotoxicity testing showed that the aloe-emodin metabolite is a more potent inducer of micronuclei than chrysophanol. Further studies showed that aloe-emodin has anticancer activity with selectivity against neuroectodermal tumors (Pecere et al., 2000). Aloe-emodin was identified as a potential anti-tumor agent and led to pharmacokinetic studies which can be used for pre-clinical investigations (Zaffaroni et al., 2003). *Aloe* species have been investigated for their anti-inflammatory and wound healing properties (Heggers et al., 1979, Cappaso et al., 1983, Klein et al., 1988, Lindsey et al., 2002). The complexity of the inflammatory process and wound healing may be addressed by different gel components (Lindsey et al., 2002). *A. ferox* showed an 80% cyclooxygenase inhibition but the group of compounds responsible for the observed anti-inflammatory activity was not identified. Earlier studies showed that emodin, aloe-emodin and aloin can be metabolised by the Koble reaction to produce salicylates (Klein and Penneys et al., 1988). Salicylic acid is known for its analgesic and anti-inflammatory properties as has been demonstrated in *A. vera* (Robson et al., 1982). However recent studies on Cape aloe showed that aloeresin I has good anti-inflammatory activity (Speranza et al., 2005). The antimalarial drug chloroquine has also been shown to be effective as an anti-inflammatory drug (Mackenzie et al., 1983). Medicinal plants used to treat inflammation can potentially be useful against malaria. *Aloe* species have previously been investigated for *in vitro* antiplasmodial activity against *P. falciparum* but no significant activity was observed (van Zyl et al., 2002). Aloin was not active against the malaria parasite. Recently, *A. ferox* was tested for *in vitro* antiplasmodial activity and revealed several promising results (Clarkson et al., 2004).

The phytochemistry of aloes has been studied since 1851 with the first preparation of the major compound aloin from Barbados aloes (Trease and Evans et al., 1978). Extensive phytochemical investigations of *Aloe* species have led to the isolation of known as well as novel compounds. Aloes are known to contain anthraquinones, chromones aloeresins and aloesin as well as glycosides such as aloin and barbaloin (Gramatica et al., 1982, Speranza et al., 1985, Manitto et al., 2003, Speranza et al.,

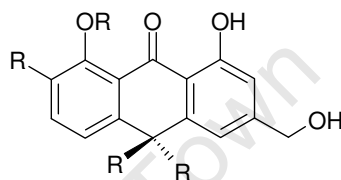


2005, Speranza et al., 1997, Bisrat et al., 2000, Dagne et al., 1997, Trease and Evans et al., 1978). Chromones and anthrones are the main secondary metabolites in *Aloe* species (Esteban-Carrasco et al., 2001). Derivatives of these compounds occur naturally in several species.

Chromone structure



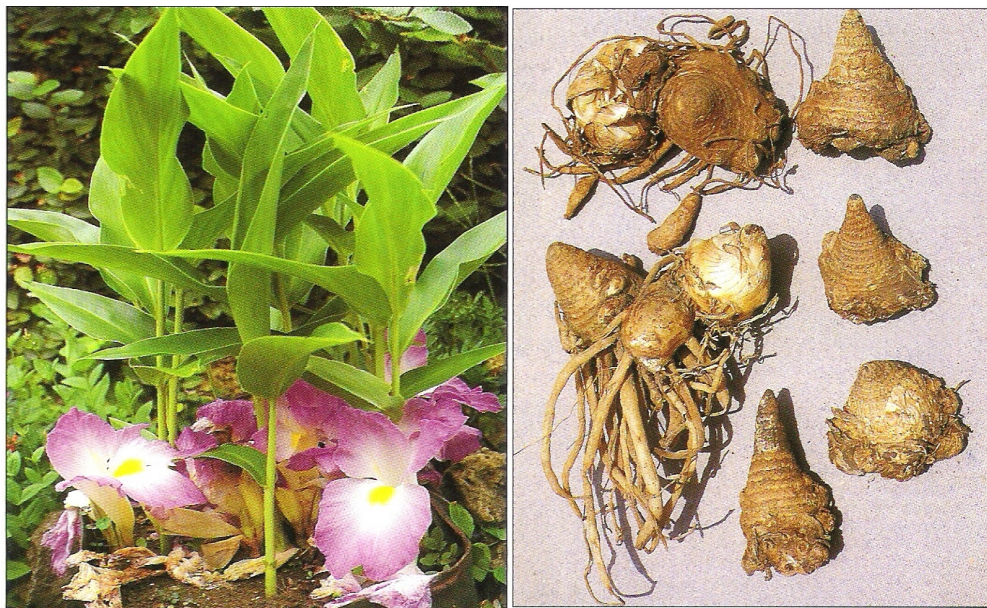
Anthrone structure



An investigation of the essential oil of the *A. ferox* leaves identified 21 compounds (Magwa et al., 2006). Leaf exudates revealed the presence of aloesin, aloeresin A, aloin A and B, aloinoside A and B, and a novel 1-methyl tetralin derivative namely feroxidin (Viljoen et al., 2001 and Speranza et al., 1990). Fresh leaf juice undergoes thermal treatment to produce the commercial bittering product, Cape Aloe. Process products play an important role in the safety, nutrition and flavour of foods. A study investigating the process products of prolonged heating resulted in the isolation of a novel benzochromanone derivative (Speranza et al., 1996). The roots of 172 *Aloe* species were investigated in a chemotaxonomic survey using chemical markers such as anthraquinones and pre-anthraquinones at an infrageneric level (van Wyk et al., 1995). The study identified the major components in *A. ferox* roots to be chrysophanol, asphodeline, aloesaponarin I and II, as well as aloesaponol I and II. Broad chemotaxonomic screens of a variety of *Aloe* species have contributed to understanding natural relationships (Viljoen et al., 2002a). Several aloes have shown chemical similarities between morphologically and geographically diverse species (Viljoen et al., 2001). The hypothesis of convergent evolution might explain this phenomenon.



## 2.2 *Siphonochilus aethiopicus*



Leaves, flowers and rhizomes of *S. aethiopicus*. Photographs obtained from Medicinal Plants of South Africa (van Wyk et al., 1997)

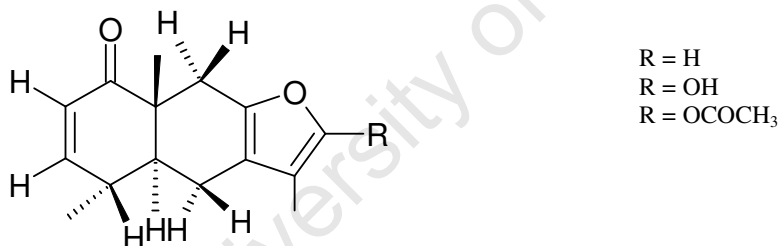
*Siphonochilus aethiopicus* (Schweinf) B.L Burt belongs to the Zingiberaceae family (van Wyk, et al., 1997). The Zingiberaceae family has 49 genera and 1 300 species consisting of perennial aromatic herbs with fleshy rhizomes and tuberous roots (Trease and Evans et al., 1978). The family is known for its volatile oils and pungent characteristics. *S. aethiopicus* was previously described as a *Kaempferia* sp. known as *K. aethiopica* or *K. ethelae* (Hutchings et al., 1996). Plants which are known for their culinary purposes like ginger (*Zingiber officinale*) and turmeric (*Curcuma longa*) belong to the Zingiberaceae family (Watt and Breyer-Brandwijk et al., 1962). Turmeric is used as traditional medicine to treat several ailments such as headache jaundice, skin disease and hepatitis throughout Africa, India and Mauritius, and is also cultivated in India, West Pakistan, China and Malaysia (Trease and Evans et al., 1996; Watt and Breyer-Brandwijk et al., 1962). Ginger is traditionally used to treat headache, rheumatism, and cough. It is also used as a mosquito repellent and wound dressing. Varieties of the ginger grow in Africa, Jamaica, China and India. The rhizomes and leaves of *S. aethiopicus* have a similar smell to that of ginger but no further similarities have been observed (van Wyk et al., 1996). Fresh rhizomes and

roots are chewed as traditional medicine. During the growing season rhizomes and roots are used while in winter the rhizomes are used without roots or leaves. The rhizomes are traditionally used to treat colds, coughs, influenza, hysteria, pain, asthma and dysmenorrhoea. *S. aethiopicus* is used by the Swazi to treat malaria and chewed by women to relieve pain during menstruation (Hutchings et al., 1996; Watt and Breyer-Brandwijk et al., 1962). Cold rhizome infusions are administered to horses against horse sickness. An overdose can stupefy horses. Wild ginger was identified as one of the most frequently used medicinal plants in South Africa (Stafford et al., 2005). The harvesting of rhizomes for traditional medicine has resulted in it almost being extinct in the wild (Zschocke et al., 2000). There is growing scientific support to conserve endangered medicinal plants using plant part substitution and to investigate genetic diversity for cultivation purposes (Zschocke et al., 2000, Viljoen et al., 2002b, Makhuvha et al., 1997). Wild ginger is easily cultivated in warm parts of South Africa and attempts at large-scale production of rhizomes using tissue culture is being researched (van Wyk, et al., 1997). The chemical profile and essential oil composition of rhizomes and roots are almost identical (Zschocke et al., 2000 and Viljoen et al., 2002b). These studies encourage the use of roots and the replanting of rhizomes to conserve this important medicinal plant. Scientific research has played a major role in conservation and cultivation. Cultivated plants have decreased the pressure imposed on wild populations.

*S. aethiopicus* was previously scientifically investigated for some of its traditional uses. The ethanol extract of the rhizomes has shown activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli* and *Klebsiella pneumoniae* (Stafford et al., 2005). The ethanol extract of the leaves and tubers or stems showed better anti-inflammatory activity than the roots and rhizomes (Lindsey et al., 1999 and Zschocke et al., 2000). However the underground parts of the plant are mostly used for traditional medicine. Interestingly the ethanol extract of the rhizomes showed an increase in anti-inflammatory and antibacterial activity after 90 days of storage (Stafford et al., 2005). The water extracts were less active after storage which might explain why traditional healers prepare fresh stocks. Anti-inflammatory studies revealed that *S. aethiopicus* inhibits the prostaglandin biosynthesis pathway but has no effect on uterine contractions (Lindsey et al., 1999). The plant is considered to be

potentially useful to treat dysmenorrhoea. The rhizomes of mature plants showed significantly higher anti-inflammatory activity than younger smaller plants (Zschocke et al., 2000). Thus plants may have to be a certain age or size for harvesting.

Although wild ginger is widely used limited information is available about its phytochemistry. Attempts at isolating anti-inflammatory compounds revealed that the activity of the crude extract might be due to synergism (Jäger et al., 2005). Galenic preparations may not always have the same clinical response as isolated drugs (Trease and Evans et al., 1978). Synergism may be due to the complex nature of plant extracts. A chemotaxonomic survey led to the isolation of two novel furanoterpenoids from *S. aethiopicus* (Holzapfel et al., 2002). The major compound 4 $\alpha$ H-3,5 $\alpha$ ,8 $\beta$ -trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8-one was named siphonochilone and represents 20 – 30% of the essential oil composition of rhizomes and roots (Viljoen et al., 2002b). Wild ginger has been scientifically investigated for its use as an anti-septic, pain-reliever and use during menstruation. However, to our knowledge, no antimalarial studies have been performed on this medicinal plant.



Chemical structures of compounds isolated or derived from *S. aethiopicus* (Holzapfel et al., 2002)

# *Chapter 3*

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*Isolation and characterization of  
compounds from S. aethiopicus*

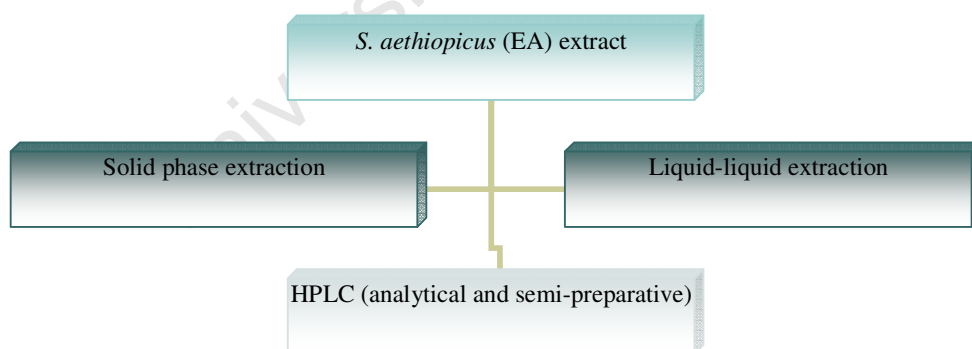
### 3. Isolation and characterization of compounds from *S. aethiopicus*

#### 3.1 Introduction

*S. aethiopicus* was investigated for the presence of novel antiplasmodial compounds using chromatographic techniques such as solid phase extraction (SPE), liquid-liquid extraction and high performance liquid chromatography (HPLC). The extracts and compounds were screened for *in vitro* antiplasmodial activity and cytotoxicity (Chapter 7). Compounds were also tested for antimycobacterial, antibacterial and antifungal activity. Structure elucidation was determined using spectral data such as  $^1\text{H}$ ,  $^{13}\text{C}$ , HSQC, HMQC, COSY and NOESY NMR spectra as well as HR-EI-MS spectra.

#### 3.2 Bioassay-guided fractionation

The rhizomes of *S. aethiopicus* were extracted with ethyl acetate (EA) and the yields determined (3.5%). *S. aethiopicus* was fractionated using a combination of chromatographic techniques. The fractionation process is depicted in Scheme 2.



Scheme 2 Fractionation procedure of *S. aethiopicus* extract

The EA extract was fractionated using SPE methods (7.3.1). Enhancement in activity was observed with the 50% to 80% acetonitrile (ACN) SPE fractions compared to the

crude extract (Fig 6). The concentration inhibiting 50% of growth ( $IC_{50}$ ) is given as a mean value of 4 independent experiments.

### SPE fractions against *P.falciparum* D10 strain

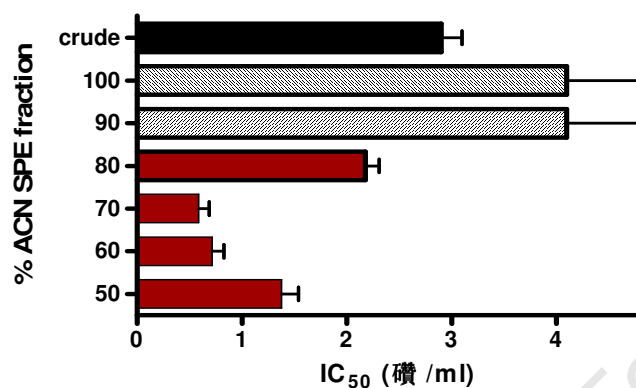


Figure 6 Averaged  $IC_{50}$ -values of SPE fractions against the CQS D10 strain of *P. falciparum*

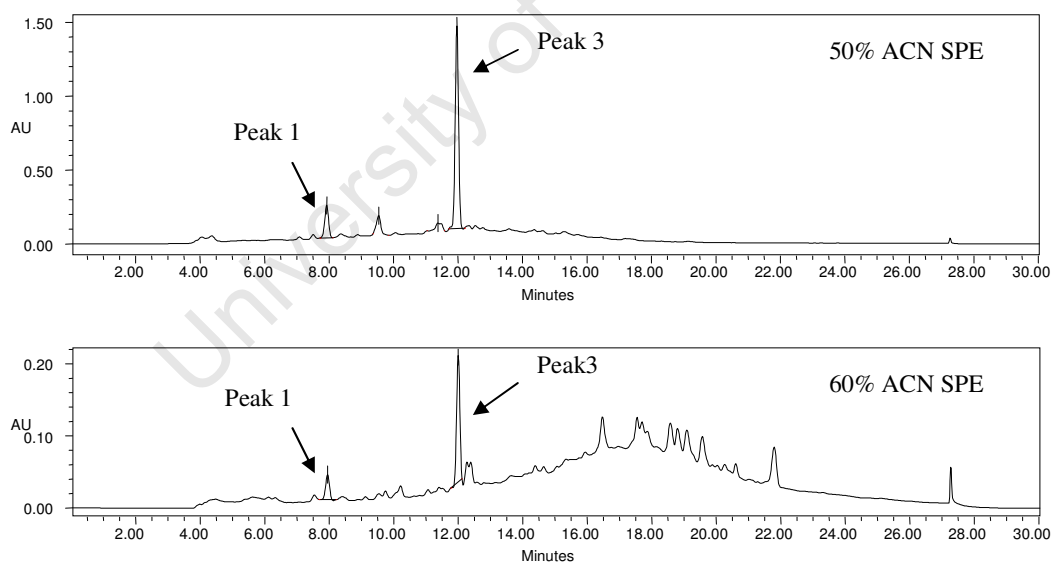


Figure 7 HPLC profiles of SPE fractions on a C18 RP column ( $\lambda = 254$  nm)

HPLC conditions: Mobile phase ACN:H<sub>2</sub>O using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

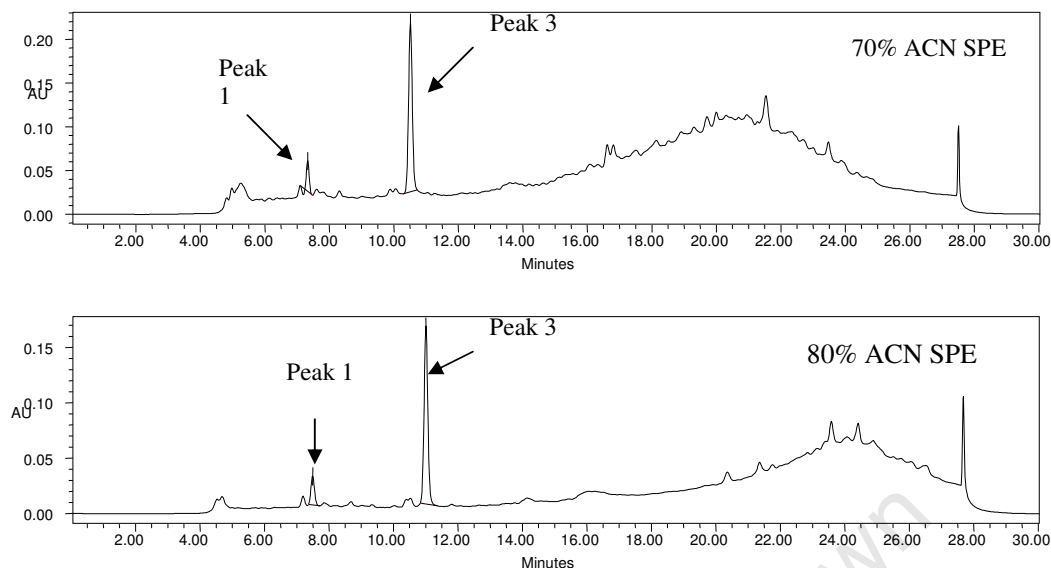


Figure 8 Continued HPLC profiles of SPE fractions

Two major peaks were present in the HPLC profiles of the SPE fractions (Fig 7 and 8). Fractionation of the crude extract was optimised via liquid-liquid extraction by using EA and a range of pH buffers described in Chapter 7 (7.3.2). This fractionation process was not as time consuming as SPE.

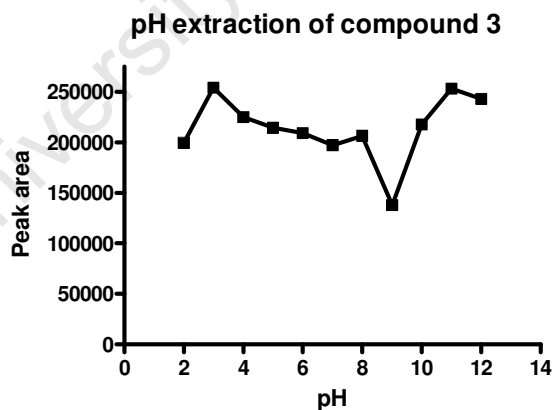


Figure 9 HPLC analyses of pH extractions of compound 3

pH 3 and 11 showed good enhancement in extraction of the active peak (Fig 9). The acidic nature of pH 3 was undesirable for large scale extraction as compounds might be unstable at this pH. pH 11 was selected for large scale extraction. Further purification was done using semi-preparative HPLC (Fig 10).

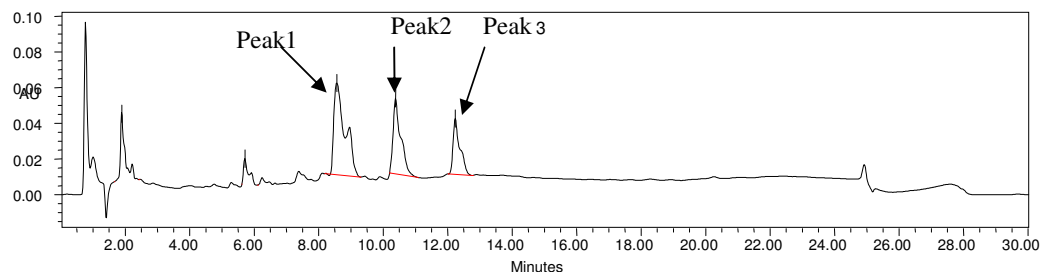


Figure 10 HPLC profile of liquid-liquid fraction on a C18 RP column ( $\lambda = 254$  nm)  
HPLC conditions: Mobile phase  $\text{H}_2\text{O}:\text{ACN}$  using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

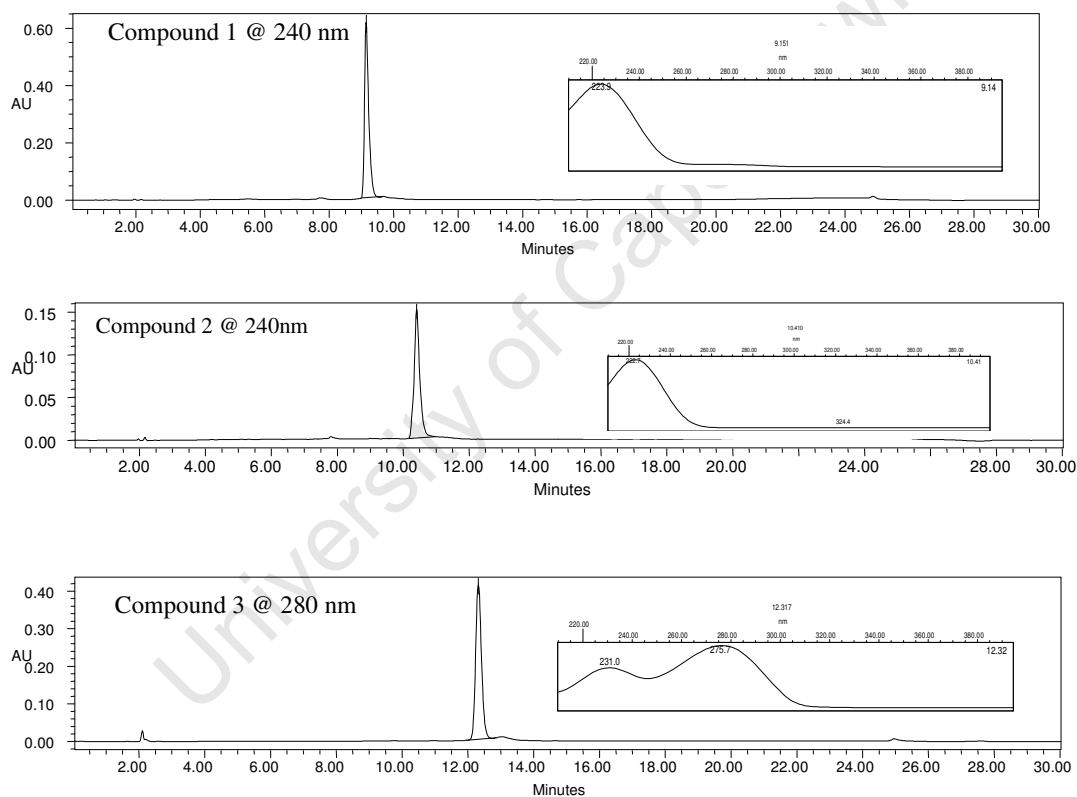


Figure 11 HPLC profiles and UV absorption spectra of compounds 1 – 3

The SPE fractions were purified via semi-preparative HPLC and resulted in the isolation of three peaks (Fig 11). The structure elucidation of isolated compounds (1 – 3) was determined using 1D and 2D NMR as well as HR-APCI-MS analyses.



## 3.3 Structure elucidation of compounds 1 – 3

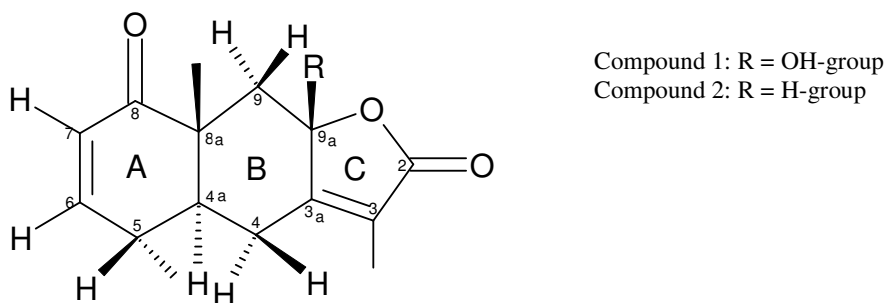
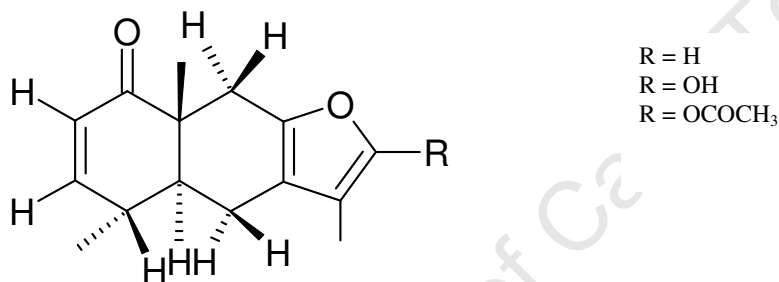


Figure 12 Chemical structures of compounds 1 and 2.

Figure 13 Chemical structures of compounds previously isolated and derived from *S. aethiopicus*

Previous studies have led to the isolation of two furanoterpenoid derivatives namely, 4 $\alpha$ H-3,5 $\alpha$ ,8 $\beta$ -trimethyl-4,4a,9-tetrahydro-naphtho[2,3-*b*]-furan-8-one and 2-hydroxy-4 $\alpha$ H-3,5 $\alpha$ ,8 $\beta$ -trimethyl-4,4a,9-tetrahydronaphtho[2,3-*b*]-furan-8-one, from *S. aethiopicus* (Holzapfel et al., 2002). In this study a further three novel compounds were isolated from this plant. To our knowledge this is the first time that these compounds have been isolated. The compounds were characterised by <sup>1</sup>H, <sup>13</sup>C, HSQC, HMQC, NOESY, COSY and HR-APCI-MS data. The spectral data for rings A and B compared well with previously published data but significant differences were observed for ring C (Fig 12 and Fig 13).

Table 1 <sup>1</sup>H, <sup>13</sup>C, HSQC, HMQC, NOESY and COSY spectral data of compound1

Position		$\delta$ H (ppm)	$\delta$ C (ppm)	HMQC	NOESY	COSY
2			171.6 <i>s</i>			
3			122.4 <i>s</i>			
3a			160.1 <i>s</i>			
4	$\alpha$	2.90 <i>dd</i> (13.3; 3.6 Hz)	25.3 <i>t</i>	C-3; C-3a; C-4a; C-9a	CH <sub>3</sub> -3; CH <sub>3</sub> -5	H-4a; H-4 $\beta$
	$\beta$	2.36 <i>dd</i> (13.5; 13.3 Hz)		C-3; C-3a; C-4a	H-5 $\beta$ ; CH <sub>3</sub> -8a	H-4a; H-4 $\alpha$
4a		1.62 <i>ddd</i> (9.0; 9.0; 3.6 Hz)	51.1 <i>d</i>	C-8; C-8a; CH <sub>3</sub> -8a		H-4a; H-4 $\beta$ ; H-5 $\beta$
5	$\beta$	2.53 <i>tq</i> (7.2; 2.4 Hz)	35.3 <i>d</i>	C-4a; C-8a	CH <sub>3</sub> -8a	H-4a; CH <sub>3</sub> -5
6		6.74 <i>dd</i> (10.2; 1.8 Hz)	156.0 <i>d</i>	C4a; CH <sub>3</sub> -5		H-7
7		5.81 <i>dd</i> (10.2; 3.0 Hz)	126.8 <i>d</i>			H-6
8			204.3 <i>s</i>			
8a			46.4 <i>s</i>			
9	$\alpha$	2.49 <i>d</i> (14.1 Hz)	44.8 <i>t</i>	C-3a; C-4a; C-8a; C-9a; CH <sub>3</sub> -8a		H-9 $\beta$
	$\beta$	1.60 <i>d</i> (14.1 Hz)		C-8; C-8a; C-9a; CH <sub>3</sub> -8a		H-9 $\alpha$
9a			104.5 <i>s</i>			
3-Me		1.78 <i>d</i> (1.2 Hz)	8.9 <i>q</i>	C-2; C-3; C-3a	H-4 $\alpha$	
5-Me		1.22 <i>d</i> (7.2 Hz)	18.9 <i>q</i>	C-4a; C-5; C-6	H-4 $\alpha$	H-5 $\beta$
8a-Me		1.30 <i>s</i>	17.8 <i>q</i>	C-4a; C-8; C-8a; C-9	H-4 $\beta$ ; H-5 $\beta$ ; 9a-OH	
9a-OH		4.84 <i>br s</i>			CH <sub>3</sub> -8a	

### 3.3.1 Compound 1

Compound 1 named 9 $\alpha$ -Hydroxy 4 $\alpha$ H-3,5 $\alpha$ ,8 $\alpha$ -trimethyl-4,4 $\alpha$ ,8 $\alpha$ ,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one was obtained as a pale yellow solid  $[\alpha]_D^{20} = -8.9^\circ (c.1.0, MeOH)$ , melting point 179.1°C (yield from EA extract = 4.39%). The molecular formula was established as C<sub>15</sub>H<sub>18</sub>O<sub>4</sub> from the [M+1]<sup>+</sup> ion at 263.1278 in the HR-APCI-MS data (calculated mass m/z 263.1283). Investigation of the MS data revealed the most abundant fragment at a mass of m/z 245.1200, which indicated a loss of an H<sub>2</sub>O-group. The NMR results for compound 1 are summarised in Table 1.

Ring A: The HMQC spectrum identified quaternary carbons C-2 ( $\delta$  171.6), C-3 ( $\delta$  122.4), C-3a ( $\delta$  160.1), C-8 ( $\delta$  204.3) and C-8a ( $\delta$  46.3). The structure determination was initiated at the most deshielded proton H-6, resonating at  $\delta$  6.74 as a double-doublet ( $J=10.2\text{Hz}; 1.8\text{Hz}$ ). This chemical shift is indicative of an aromatic proton or conjugated double bond system. Coupling constants of 10.2Hz are typical of vicinal-coupling across a double bond or ortho-coupling in an aromatic system. The coupling constant at H-6 of 1.8Hz can be attributed to long range coupling. A second proton with a coupling constant of 10.2Hz was identified as H-7 ( $\delta$  5.81). The COSY spectrum confirmed that the protons at  $\delta$  6.74 and  $\delta$  5.81 are coupled. The chemical shift at  $\delta$  5.81 is too shielded for an aromatic proton thus suggesting a carbon-carbon double bond in a non-aromatic system. An aromatic system was thus eliminated as a possibility. The large coupling constant of 10.2Hz can be accounted for by vicinal-coupling.

Although COSY correlations suggest a *d* splitting pattern at H-7 the multiplicity appears as a *dd* due to long range allylic coupling with H-5 ( $J=3\text{Hz}$ ) (Lambert et al., 1998). The HSQC spectrum identified the carbon resonating at  $\delta$  126.8 as C-7, and the carbon at  $\delta$  156.0 as C-6. The chemical shifts of the carbons C-6 and C-7 are typical of a  $\alpha,\beta$ -unsaturated carbonyl system (Breitmaier et al., 1987). In the  $\alpha,\beta$ -unsaturated carbonyl system C-6 is the deshielded  $\beta$ -carbon and C-7 is the  $\alpha$ -carbon (Lambert, 1998). H-6 is coupled to C-4a ( $\delta$  51.1) and C-5Me ( $\delta$  18.9) in the HMQC spectrum. The chemical shift of  $\delta$  18.9 is typical of a methyl carbon. The COSY spectrum

identified a triple quartet resonating at  $\delta$  2.53 ( $J=7.2\text{Hz}$ ;  $2.4\text{Hz}$ ) as H-5. Complementary evidence from the HSQC and COSY spectra confirmed that the carbon at  $\delta$  35.5 is C-5. The NOESY spectrum coupled H-5 to methyl protons resonating as a singlet at  $\delta$  1.30 (8aMe) indicating that the protons have the same spacial orientation. The  $d$  resonating at  $\delta$  1.22 ( $J=7.2\text{Hz}$ ) was assigned to 5Me in the HSQC spectrum. The HMQC data linked 5-Me to C-4a ( $\delta$  51.1) and C-5. Evidence from the NOESY spectrum revealed coupling between 5-Me and H-4 $\alpha$ . This indicates that 5-Me has the same spacial orientation as H-4 $\alpha$ . The COSY spectrum also coupled H-5 to 5-Me and H-4a resonating at  $\delta$  1.62 ( $J=9.0\text{Hz}$ ;  $9.0\text{Hz}$ ;  $3.6\text{Hz}$ ). The multiplicity at H-4a appears as a *ddd* due to splitting by H-5, H-4 $\alpha$  and H-4 $\beta$  as revealed by coupling shown in the COSY spectrum. The HSQC spectrum identified the carbon at  $\delta$  51.1 as C-4a. The NOESY spectrum coupled H-4a to 5Me. The orientation of these protons is  $\alpha$ . The HMQC spectrum coupled C-4a to a carbonyl carbon resonating at  $\delta$  204.3 assigned as C-8.

Ring B: The COSY spectrum coupled H-4a to H-4 $\alpha$  at  $\delta$  2.90 ( $J=13.2\text{Hz}$ ;  $3.6\text{Hz}$ ) and H-4 $\beta$  at  $\delta$  2.35 ( $J=13.2\text{Hz}$ ;  $3.6\text{Hz}$ ). Supporting evidence from the HSQC spectrum coupled these protons to the same carbon at  $\delta$  25.3 identified as C-4. Geminal ( $J=13.2\text{Hz}$ ) and vicinal ( $J=3.6\text{Hz}$ ) coupling suggests *dd* multiplicities for both H-4 $\alpha$  and H-4 $\beta$ . However the multiplicity at H-4 $\beta$  appeared as overlapping *dd*'s not clearly defined. The COSY spectrum coupled H-4 $\alpha$  to H-4 $\beta$  and both to H-4a. The assignment of H-4 $\alpha$  was further supported by the NOESY correlations to 3Me and 5Me. The HMQC spectrum showed correlations between H-4 $\alpha$  and H-4 $\beta$  to C-3, C-3a, and C-4a and in addition H-4 $\alpha$  also coupled to C-9a. The methyl group resonating as a singlet at  $\delta$  1.30 was assigned as 8a-Me. Orientation of 8aMe in the  $\beta$ -position was supported by coupling to H-4 $\beta$  and H-5 in the NOESY spectrum. Complimentary evidence from the HMQC spectrum linked 8a-Me to C-4a, C-8, C-8a and C-9. This further supported the assignment of 8a-Me in the molecule.

The HSQC spectrum linked H-9 $\alpha$  and H-9 $\beta$  to the same carbon at  $\delta$  44.8 (C-9). H-9 $\alpha$  was assigned to the  $d$  resonating at  $\delta$  2.49 with a geminal coupling constant of  $J=14.1\text{Hz}$ . Complementary evidence was obtained from the HMQC spectrum which correlated H-9 $\alpha$  to C-3a, C-4a, C-8a, 8a-Me and C-9a. The COSY spectrum linked H-

9 $\alpha$  to a *d* resonating at  $\delta$  1.6 ( $J=14.1\text{Hz}$ ) identified as H-9 $\beta$ . Further evidence from the HMQC spectrum correlated H-9 $\beta$  to C-8, C-8a, 8a-Me and C-9a.

**Ring C:** A second  $\alpha$ ,  $\beta$ -unsaturated carbonyl system was identified at C-3a ( $\delta$  160.1) and C-3 ( $\delta$  122.4). C-3a is the deshielded  $\alpha$ -carbon and C-3 the  $\beta$ -carbon. 3Me ( $\delta$  8.9) coupled to C-3, C-3a and C-2 in the HMQC spectrum. The attachment of 3-Me to C-3 confirms that C-3 is a methyl constituent at the  $\beta$ -carbon in the  $\alpha$ ,  $\beta$ -unsaturated carbonyl system (Lambert, 1998). 3-Me resonated as a *d* at  $\delta$  1.78 ( $J=1.2\text{Hz}$ ) and this multiplicity can be accounted for by long range allylic coupling with H-4 $\alpha$  as revealed in the COSY spectrum. The location of the OH-group was convincingly established at C-9a based on a molecular model as well as the conversions of compounds 1 to 2 and 2 to 3.

### 3.3.2 Compound 2

Compound 2 named 4 $\alpha$ H-3,5 $\alpha$ ,8 $\alpha\beta$ -Trimethyl-4,4a,8a,9-tetrahydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one was obtained as a yellow oily solid  $[\alpha]_D^{22} = +3.39^\circ (c.1.0, MeOH)$ , melting point  $60^\circ\text{C}$  (yield from EA extract = 2.62%). Compound 2 gave a  $[M+H]^+$  ion in the HR-APCI-MS data at 247.1340, which corresponded to the molecular formula of  $C_{15}H_{18}O_3$  (calculated mass  $m/z$  247.1334). The NMR data of compound 2 was comparable to compound 1 for ring A and B but differences were observed in ring C (Fig 12 and Table 2).

**Ring C:** A difference of 16D between  $M_1^+$  and  $M_2^+$ , indicated the replacement of the OH group at C-9a by an H atom. This was confirmed by the appearance of a *dd* at  $\delta$  4.9 coupled in the COSY spectrum to the H-9 $\alpha$  and H-9 $\beta$  *dd*'s at  $\delta$  2.66 and  $\delta$  1.62, respectively (Table 2). Further confirmation came from the correlation between H-9a and C-3a in the HMQC spectrum. Finally a NOESY correlation between H-9a and 8aMe established its  $\beta$ -orientation.

Table 2  $^1\text{H}$ ,  $^{13}\text{C}$ , HSQC, HMQC, NOESY and COSY spectral data of compound2

Position		$\delta\text{H}$ (ppm)	$\delta\text{C}$ (ppm)	HMQC	NOESY	COSY
2			174.2 s			
3			119.7 s			
3a			161.0 s			
4	$\alpha$	3.03 <i>dd</i> (14.3; 4.8 Hz)	25.2 <i>t</i>	C-3; C-3a; C-4a; C-5; C-8a; C-9	$\text{CH}_3$ -3; $\text{CH}_3$ -5	H-4 $\beta$ ; H-4a
	$\beta$	2.31 <i>dd</i> (14.3; 12.0 Hz)		C-3; C-3a; C-4a; C-5	H-5 $\beta$ ; $\text{CH}_3$ -8a	H-4 $\alpha$ ; H-4a
4a		1.64 <i>dd</i> (13.8; 4.2 Hz)	48.1 <i>d</i>	C-4; C-5; $\text{CH}_3$ -5; C-8; C-8a; $\text{CH}_3$ -8a		H-4a; H-4 $\beta$ ; H-5 $\beta$
5	$\beta$	2.50 <i>tq</i> (7.2; 2.4 Hz)	36.4 <i>d</i>		H-4 $\beta$ ; $\text{CH}_3$ -8a	H-4a; $\text{CH}_3$ -5
6		6.76 <i>dd</i> (10.2; 1.8 Hz)	154.5 <i>d</i>	C-4a; C-5; $\text{CH}_3$ -5; C-8		H-7
7		5.84 <i>dd</i> (10.2; 2.4 Hz)	125.3 <i>d</i>	C-5; C-8a		H-6
8			202.2 s			
8a			44.9 s			
9	$\alpha$	2.66 <i>dd</i> (12.9; 6.0 Hz)	39.6 <i>t</i>	C-3a; C-4a; C-8a; $\text{CH}_3$ -8a; C-9a		H-9 $\beta$
	$\beta$	1.63 <i>dd</i> (12.9; 4.2 Hz)		C-3a; C-4a; C-8; C-8a; $\text{CH}_3$ -8a		H-9 $\alpha$
9a		4.90 <i>dd</i> (16.0; 6.6 Hz)	77.8 <i>d</i>	C-3a	$\text{CH}_3$ -8a	H-9 $\alpha$ ; H-9 $\beta$
3-Me		1.78 <i>d</i> (1.2 Hz)	7.4 <i>q</i>	C-2; C-3; C-3a	H-4 $\alpha$	
5-Me		1.21 <i>d</i> (7.2 Hz)	17.3 <i>q</i>	C-4a; C-5; C-6	H-4 $\alpha$	H-5 $\beta$
8a-Me		1.31 <i>s</i>	15.3 <i>q</i>	C-4a; C-8; C-8a; C-9	H-4 $\beta$ ; H-5 $\beta$	

### 3.3.3 Compound 3

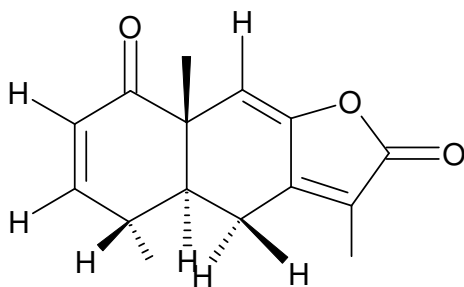


Figure 14 Chemical structure of compound 3

Compound 3 named 4aH-3,5α,8aβ-Trimethyl-4,4a,8a-trihydronaphtho-([2,3b]-dihydrofuran-2-one)-8-one was obtained as a yellow solid  $[\alpha]_D^{22} = +2.37^\circ (c.1.0, MeOH)$ , melting point  $77^\circ\text{C}$  (yield from EA extract = 1.89%). The molecular formula,  $\text{C}_{15}\text{H}_{16}\text{O}_3$ , was deduced from the  $[\text{M}+\text{H}]^+$  ion at 245.1161 in the HR-APCI-MS data (calculated 245.1178). The NMR data of compound 3 was comparable to compounds 1 and 2 except for differences observed with ring C (Fig 14 and Table 3).

**Ring C:** A difference of 2D between compound 2 and 3 could be attributed to the formation of an olefinic double bond between C-9 and C-9a. Confirmation came from the appearance of a proton singlet at  $\delta$  6.29 and  $2J_{\text{CH}}$  and  $3J_{\text{CH}}$  correlations from H-9 to C-9a, C-3a, C-4a and C-8 (Table 3). The additional  $\alpha,\beta,\gamma$ -conjugation resulted in a more shielded  $^{13}\text{C}$  resonance for C-2 at  $\delta$  170.0.

Table 3  $^1\text{H}$ ,  $^{13}\text{C}$ , HSQC, HMQC, NOESY and COSY spectral data of compound3

Position		$\delta\text{H}$ (ppm)	$\delta\text{C}$ (ppm)	HMQC	NOESY	COSY
2			170.9 s			
3			117.3 s			
3a			146.8 s			
4	$\alpha$	3.06 <i>dd</i> (17.4; 4.8 Hz)	22.4 <i>t</i>	C-3; C-3a; C-4a; C-9a	CH <sub>3</sub> -5	H-4 $\beta$ ; H-4a
	$\beta$	2.52 <i>dd</i> (17.4; 1.8 Hz)			CH <sub>3</sub> -8a	H-4 $\alpha$ ; H-4a
4a		2.02 <i>ddd</i> (13.2; 11.8; 4.2 Hz)	46.5 <i>s</i>	C-3a		H-4 $\alpha$ ; H-4 $\beta$
5	$\beta$	2.56 <i>tq</i> (7.5; 2.4 Hz)	33.4 <i>d</i>			H-4a; H-6; H-7
6		6.83 <i>dd</i> (10.2; 2.4 Hz)	155.1 <i>d</i>	C-4a; CH <sub>3</sub> -5		H-5; H-7; CH <sub>3</sub> -5
7		5.92 <i>dd</i> (10.2; 3.0 Hz)	125.1 <i>d</i>			H-5; H-6
8			199.8 <i>s</i>			
8a			46.6 <i>s</i>			
9		6.28 <i>s</i>	110.9 <i>d</i>	C-3a; C-4a; C-8; C-9a		CH <sub>3</sub> -3
9a			148.6 <i>s</i>			
3-Me		1.88 <i>d</i> (2.4 Hz)	7.6 <i>q</i>	C-2; C-3; C-3a		
5-Me		1.24 <i>d</i> (7.2 Hz)	17.0 <i>q</i>	C-4a; C-5; C-6	H-4 $\alpha$	H-5
8a-Me		1.92 <i>s</i>	18.1 <i>q</i>	C-4a; C-8; C-9	H-4 $\beta$ ; H-5 $\beta$	



### 3.4 Antiplasmodial activity and cytotoxicity

The crude extract and compounds were screened for *in vitro* antiplasmodial activity against the CQS D10 strain and CQR K1 strain of *P. falciparum* according to Chapter 7 (7.6). The cytotoxicity of samples was also determined against the Chinese hamster ovarian (CHO) cell-line according to methods described in Chapter 7 (7.7). Cytotoxicity was evaluated to determine the selectivity of the test samples to the test organism.

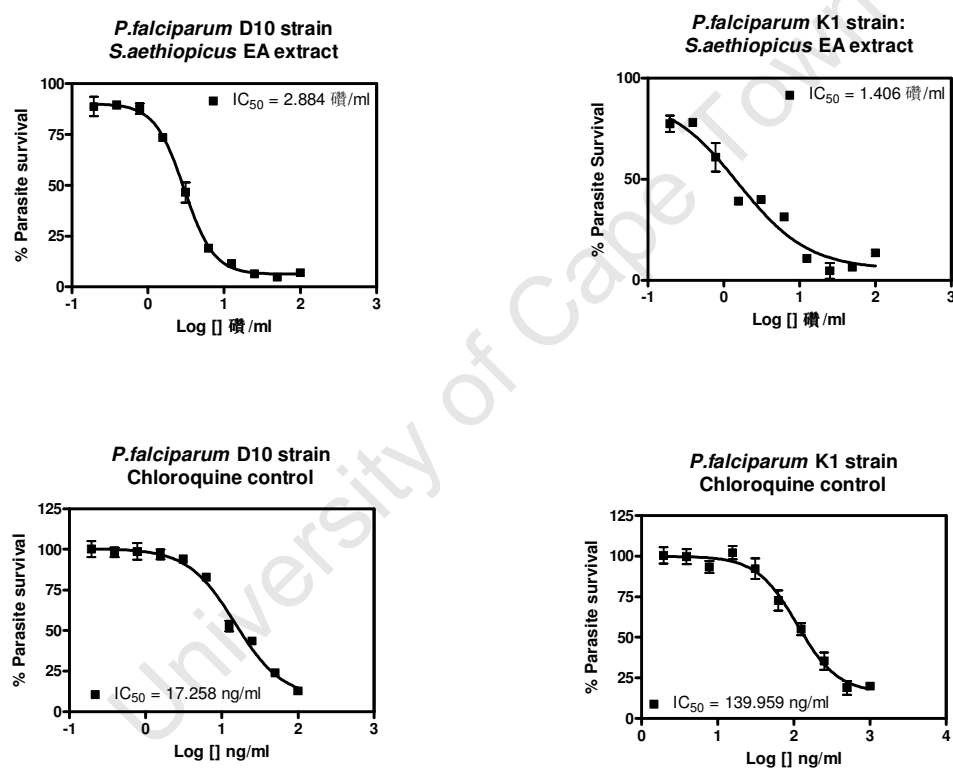


Figure 15 Pooled dose-response curves of *S. aethiopicus* extract against CQS D10 and CQR K1 strains of *P. falciparum*

The EA extract of *S. aethiopicus* showed very good *in vitro* antiplasmodial activity against the CQS D10 strain as well as the CQR K1 strain of *P. falciparum* with  $\text{IC}_{50}$ -values of 2.9  $\mu\text{g/ml}$  and 1.4  $\mu\text{g/ml}$ , respectively (Fig 15).

Table 4 *In vitro* antiparasmodial activity and cytotoxicity of the EA extract and isolated compounds

Sample	Antiplasmodial		Cytotoxicity CHO IC <sub>50</sub> (µg/ml)	SI <sup>a</sup> CHO/D10	SI <sup>b</sup> CHO/K1	RI <sup>c</sup>
	D10 IC <sub>50</sub> (µg/ml)	K1 IC <sub>50</sub> (µg/ml)				
<i>S. aethiopicus</i> extract	2.89 ±0.28	1.40 <sup>d</sup>	73.9 ±12.8	25.50	52.79	0.48
Compound 1	18.13 ±1.56	16.71 ±1.75	>100 ±ND	ND	ND	0.92
Compound 2	25.40 ±2.26	5.86 ±0.37	70.5 ±6.31	2.78	12.03	0.23
Compound 3	3.40 ±0.57	1.75 ±0.33	79.8 ±9.99	23.47	45.60	0.51
Chloroquine	10.87 x 10 <sup>-3</sup> ±3.68	146.26 x 10 <sup>-3</sup> ±30.24				13.45
Emetine			0.05 ±0.02			

IC<sub>50</sub>-values are given as a mean value with standard deviations of 2 independent experiments

Selectivity index (SI<sup>a</sup>) = cytotoxicity IC<sub>50</sub>/antiplasmodial D10 IC<sub>50</sub>

Selectivity index (SI<sup>b</sup>) = cytotoxicity IC<sub>50</sub>/antiplasmodial K1 IC<sub>50</sub>

Resistance index (RI<sup>c</sup>) = K1 IC<sub>50</sub>/D10 IC<sub>50</sub>

d = tested on one occasion in duplicate

ND = not determined

Compound 3 was the most active compound isolated with an IC<sub>50</sub>-value of 3.4 µg/ml against the CQS strain and 1.7 µg/ml against the CQR strain of the parasite (Table 4). The RI of compound 2 showed a significant increase in activity towards the CQR K1 strain. Compound 1 was equipotent against both strains of the parasite.

### 3.5 Antimycobacterial, antibacterial and antifungal activity

The antibacterial activity of *S. aethiopicus* has been established in previous studies (Stafford et al., 2005). Therefore test samples (fraction and compounds) were subjected to additional testing. Methods for activity testing are described in Chapter 7 (7.9). The liquid-liquid fraction (pH 11 buffer) consisting of a combination of compounds 1 – 3 and was tested in a preliminary screen for antimycobacterial activity against *M. aurum* A<sup>+</sup>. The compounds were tested against a broad spectrum of organisms namely *M. tuberculosis*, Gram positive *S. aureus*, Gram negative *K. pneumoniae* and the fungus *Candida albicans*.

Table 5 MIC's and IC<sub>50</sub>-value in µg/ml of compounds 1 to 3 against mycobacterium, a Gram-positive and –negative bacterium and a fungus

Sample	<i>M. tuberculosis</i>	<i>M. aurum</i> (IC <sub>50</sub> )	<i>S. aureus</i>	<i>K. pneumoniae</i>	<i>C. albicans</i>
Compound 1	250		>250	>250	>250
Compound 2	250		>250	>250	>250
Compound 3	250		>250	>250	>250
Liquid-liquid fraction		385.5			
Rifampicin	2				
Nystatin					1.6 – 3.2
Ciprofloxacin			0.3 - 0.6	0.078 – 0.15	

MIC = Minimum inhibitory concentration

A MIC of 500 µg/ml was determined for the fraction based on visual growth of *M. aurum* A<sup>+</sup>. A further test was performed to establish an IC<sub>50</sub>-value. The fraction was found to be moderately active against *M. aurum* with an IC<sub>50</sub>-value of 385.5 µg/ml (Table 5). The antimycobacterial activity of compounds 1, 2 and 3 revealed an MIC-value of 250 µg/ml against *M. tuberculosis*. The compounds showed an enhancement in activity with purification. The compounds did not show antibacterial or antifungal activity at 250 µg/ml.

### 3.6 Discussion

#### 3.6.1 Antiplasmodial activity

The EA extract of *S. aethiopicus* was active against the CQS and CQR strains of *P. falciparum*. The crude extract was subjected to bioassay-guided fractionation and yielded three novel structurally-related furanoterpenoids. Once the peaks of interest were identified the primary fractionation process was optimised. Optimisation using liquid-liquid separation instead of SPE proved to be simpler and more rapid. The most active SPE fractions namely the 50%, 60%, 70% and 80% ACN fractions were more active than the isolated compounds. These results indicated that the antiplasmodial activity of extracts of the plant might be due to synergism. This hypothesis was

supported by a recent study which deduced that the anti-inflammatory activity of *S. aethiopicus* might be due to synergism (Jäger et al., 2005). It is possible for a fraction to show significant bioactivity which might not be revealed by a single constituent of the fraction (Trease and Evans et al., 1978).

The activity of the EA extract and compound 3 is comparable against both CQS and CQR strains of *P. falciparum*. All compounds showed low cytotoxicity. The SI of compound 3 and the crude extract were also similar. The *in vitro* results suggest that the activity of the crude extract is attributable to compound 3. However the synergistic effect of different components could not be ruled out due to the activity of the SPE fractions. Compound 3 was the most active against both CQS and CQR strains of the parasite but was considered moderately active. Compounds 1 and 2 showed low activity against *P. falciparum*. Interestingly, the test samples were more selective towards the CQR strain than the CQS strain. This class of compounds is unlike current antimalarial drugs which makes it an attractive candidate for chemical modifications.

### 3.6.2 Antimycobacterial, antibacterial and antifungal activity

*S. aethiopicus* has shown activity against *B. subtilis*, *S. aureus*, *E. coli* and *K. pneumoniae* with activities ranging from 1.56 mg/ml to 3.13 mg/ml (Stafford et al., 2005). Based on these promising results additional antimycobacterial, antibacterial and antifungal testing was performed with a fraction and isolated compounds. The semi-pure fraction consisting of all three compounds was extracted using a pH 11 buffer. The fraction showed moderate activity against *M. aurum*. Screening against *M. aurum* serves as a preliminary screen for *M. tuberculosis*. Thus the isolated compounds were subjected to *in vitro* testing against *M. tuberculosis*, *S. aureus*, *K. pneumoniae* and *C. albicans*. The isolated compounds showed an MIC-value of 250 µg/ml against *M. tuberculosis*. The compounds did not show antibacterial or antifungal activity at 250 µg/ml. The fraction and isolated compounds were more active than the published results for the crude extract. However no significant activity was found with the isolated compounds against *M. tuberculosis*, *S. aureus*, *K. pneumoniae* and *C. albicans*.

# *Chapter 4*

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*In vivo study of S. aethiopicus*

#### 4. *In vivo* study of *S. aethiopicus*

The EA extract of the rhizomes of *S. aethiopicus* showed very promising *in vitro* activity against both CQS and CQR strains of *P. falciparum* (Table 4). The *in vitro* results revealed that the crude extract and isolated compounds were more selective towards the CQR strain than the CQS strain of the parasite. No *in vitro* cytotoxicity was observed with the EA extract and isolates. These interesting results lead to the investigation of *S. aethiopicus* for *in vivo* antiparasmodial activity, bioavailability and metabolism.

##### 4.1 Antiplasmodial testing

###### 4.1.1 Schizontocidal activity

The EA extract of *S. aethiopicus* rhizomes was tested in a malaria mouse-model according to methods described in Chapter 7 (7.10). The increased selectivity observed towards the CQR strain *in vitro* resulted in the selection of the CQR *P. yeolii* NS strain for *in vivo* testing. The EA extract and compound tested dissolved well in the preparations used and were administered by gavage. The controls for the vehicles used for dose preparation had no effect on parasitemia (Fig 16).

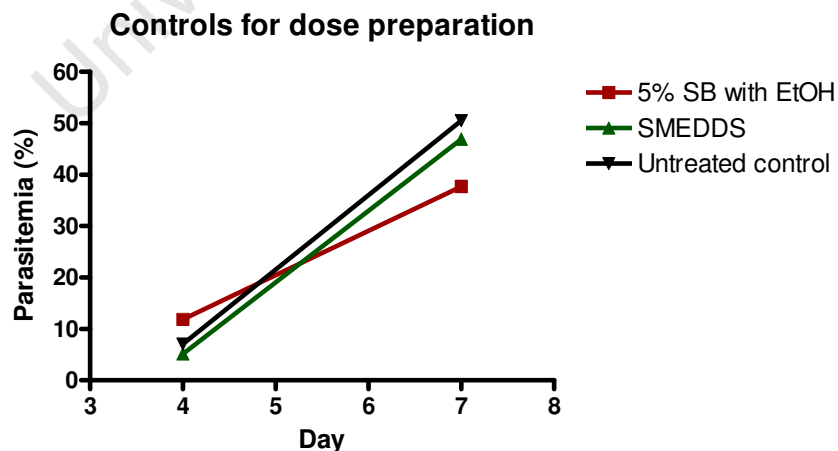


Figure 16 Solvent controls against the CQR *P. yeolii* NS strain using the 4-day suppressive test

A four-day (4-D) suppressive test was performed with the EA extract at a dose of 400 mg/kg bodyweight (bw) dissolved in 5% sodium bicarbonate (SB). The test animals were grouped into three groups of three.

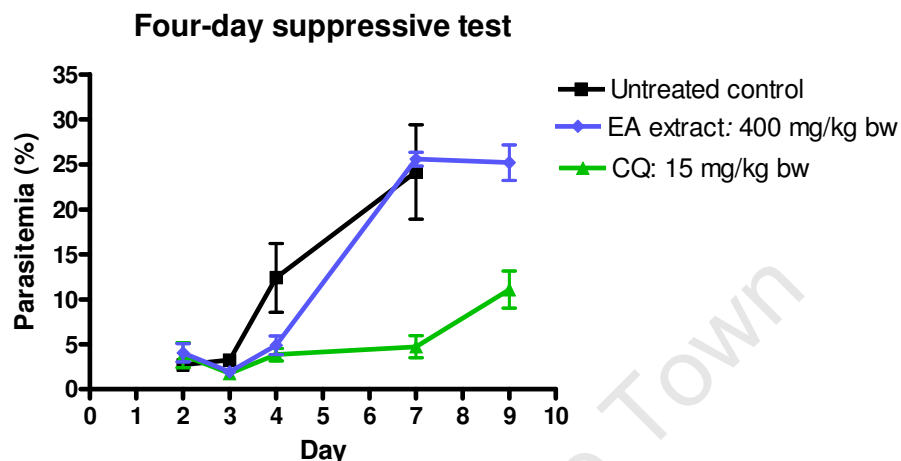


Figure 17 EA extract against CQR *P. yeolii* NS strain using the 4-day suppressive test

The study showed that the crude extract suppressed the parasitemia during treatment (Fig 17). The parasitemia rapidly increased after termination of treatment on day 4 and was comparative to the untreated control on day 7. The crude extract inhibited parasite growth by 65% on day 4 (Table 6). A decline in weight was observed over 9 days (Table 7).

Table 6 EA extract: Percentage growth inhibition against *P. yeolii* NS strain using the 4-day suppressive test

Days post-infection	<i>S. aethiopicus</i> (EA)	CQ control
3	40.51%	45.64%
4	65.13%	72.48%

Table 7 EA extract: Average weights of mice using the 4-day suppressive test against CQR *P. yoelii* NS strain ( $\pm$ SD)

Days post-infection	Untreated control	CQ control	<i>S. aethiopicus</i> (EA)
0	26.64 $\pm$ 2.31	24.36 $\pm$ 1.13	26.86 $\pm$ 0.18
1	26.74 $\pm$ 2.64	24.38 $\pm$ 0.89	26.71 $\pm$ 0.30
3	26.71 $\pm$ 1.81	24.08 $\pm$ 1.30	25.97 $\pm$ 0.40
4	26.26 $\pm$ 2.47	25.17 $\pm$ 1.46	26.73 $\pm$ 0.27
7	21.39 $\pm$ 3.91	23.87 $\pm$ 0.49	23.48 $\pm$ 1.47
9		22.10 $\pm$ 0.82	20.36 $\pm$ 0.82

The results prompted the investigation of the EA extract in a seven-day (7-D) suppressive test. The 7-D suppressive test was performed with the EA extract administered at 400 mg/kg bw which was dissolved in either 5% SB (with addition of 5% ethanol) or the lipid-based self-microemulsifying drug delivery system (SMEDDS) formulation (Table 8). The SMEDDS formulation consists of the test sample, lipids, surfactants and cosurfactants which forms a fine microemulsion of oil-in-water when exposed to an aqueous environment like the gastrointestinal tract (Wu et al., 2006). For optimal solubility in the SMEDDS formulation the test samples were first dissolved in ethanol then ethyl linoleate and lastly tween 80 was added. The addition of ethanol to 5% SB solution resulted in better solubility of the EA extract. The test animals were grouped into two groups of three for controls (untreated and CQ). The two groups receiving the EA extract consisted of four mice each.

Table 8 Composition of SMEDDS formulation

Drug / excipient	Composition
Test sample	55.5 (8.55%)
Ethanol	59.4 (9.15%)
Tween 80	356.4 (54.9%)
Ethyl linoleate	178.2 (27.4%)

Percentage composition in parenthesis

The importance of drug formulation in the evaluation of *in vivo* antimalarial activity has been shown in recent studies (Peters et al., 2002). Contributing factors such as



bioavailability and pharmacokinetics must be taken into consideration when selecting a drug formulation. Hence the activity of the crude extract was evaluated in a lipid-based and aqueous formulation. The crude extract preparation in the SMEDDS formulation had no effect on suppressing parasitemia (Fig 18). These observations are probably due to the progression of the disease. The parasitemia of this group was also higher than the untreated control group on day 5 but remained steady until day 7. The 5% SB formulation showed suppression of parasitemia on day 4 and 7 of treatment. The schizontocidal activity of *S. aethiopicus* EA extract showed that the 5% SB formulation is more effective than the SMEDDS formulation. The SMEDDS formulation group appeared very sick on day 7 and had a steady weight loss (Table 9).

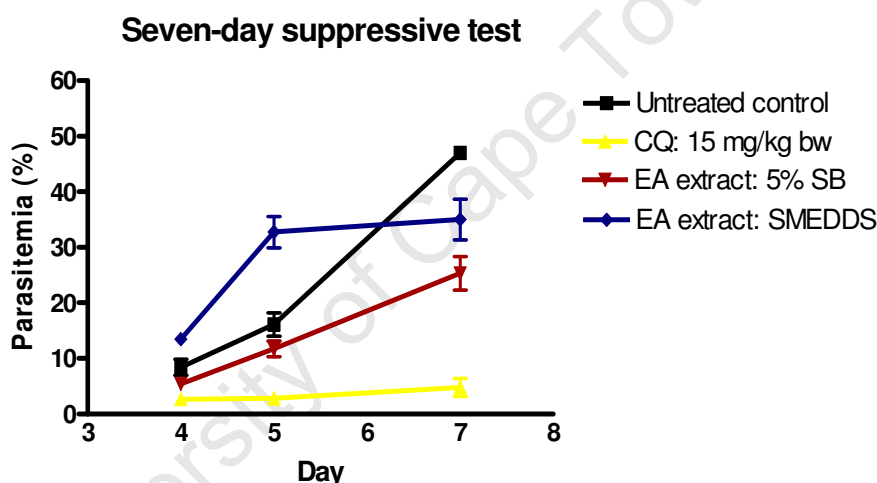


Figure 18 EA extract against CQR *P. yoelii* NS strain using the 7-day suppressive test

Table 9 EA extract: Average weights of mice using the 7-day suppressive test against CQR *P. yoelii* NS strain ( $\pm$ SD)

Days post-infection	Untreated control	CQ control	<i>S. aethiopicus</i> (EA)	
			Sodium bicarbonate	SMEDDS
0	25.40 $\pm$ 0.76	22.86 $\pm$ 2.82	25.79 $\pm$ 1.97	22.01 $\pm$ 1.11
4	23.73 $\pm$ 0.37	21.32 $\pm$ 2.58	23.75 $\pm$ 2.33	19.54 $\pm$ 2.19
6	22.30 $\pm$ 0.52	22.43 $\pm$ 2.38	22.54 $\pm$ 2.61	17.30 $\pm$ 1.27
8	19.96 $\pm$ 1.30	21.70 $\pm$ 1.71	20.18 $\pm$ 2.17	15.82 $\pm$ 0.79

Compound 3 was the most active compound isolated from the EA extract with an  $IC_{50}$ -value of 3.4  $\mu\text{g/ml}$  against the CQS D10 strain of *P. falciparum* (Table 4). Compound 3 was prepared in 10% dimethylsulphoxide (DMSO) which was established as the optimal formulation based on solubility. The compound was tested at 100 mg/kg bw using the 4-D suppressive test. Limited material allowed for testing at only one concentration. The test animals were divided into three groups with four mice in the untreated control group, three in the CQ control group and five in the group for the test compound.

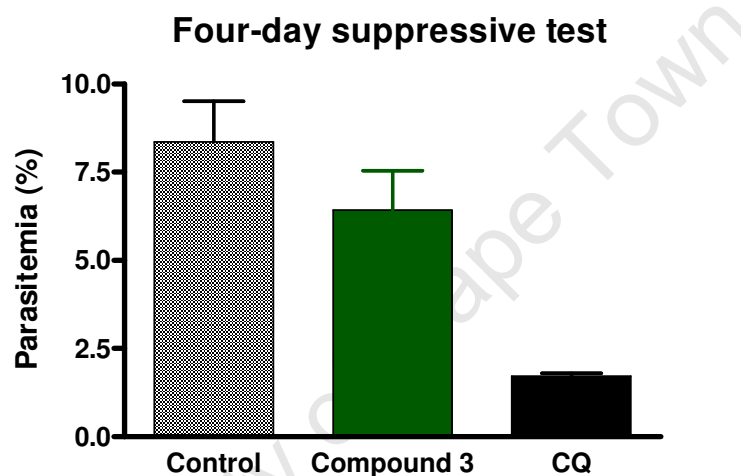


Figure 19 Compound 3: Average parasitemia on day 4 against CQR *P. yeolii* NS strain (Control = untreated)

Table 10 Compound 3: Percentage growth inhibition against *P. yeolii* NS strain on day 4

% Growth inhibition	
Compound 3	23.18
CQ	79.45

Compound 3 showed suppression of parasitemia on day 4 of treatment (Fig 19). The evaluation of the growth inhibition showed that the compound inhibited 23.2% of parasite growth on day 4 (Table 10). The compound showed minimal suppression of parasitemia compared to the control. The weight loss of the test animals decreased

significantly from day 4 to 7 for the untreated control and test compound group which indicated the severity of the disease state (Fig 20).

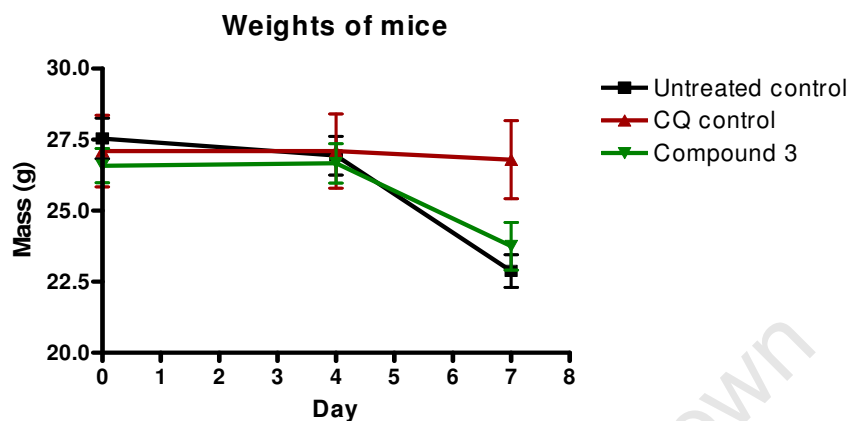


Figure 20 Compound 3: Average weights of mice using the 4-day suppressive test against the CQR *P. yoelii* NS strain

#### 4.1.2 Parasite clearance and prophylactic tests

The therapeutic effect of *S. aethiopicus* was evaluated in a parasite clearance and prophylactic experiment. The study design of the parasite clearance and prophylactic tests either started treatment four days after infection or four days before infection, respectively. The crude extract was administered by gavage at 400 mg/kg bw and 800 mg/kg bw. The extract was dissolved in 5% SB to which 5% ethanol was added. Tests were performed against the CQR *P. yoelii* NS strain.

##### 4.1.2.1 Parasite clearance test

The parasite clearance experiment was performed according to methods in Chapter 7 (7.10.4). A four day treatment schedule was started on day 4 post-infection (parasitemia of > 10%).

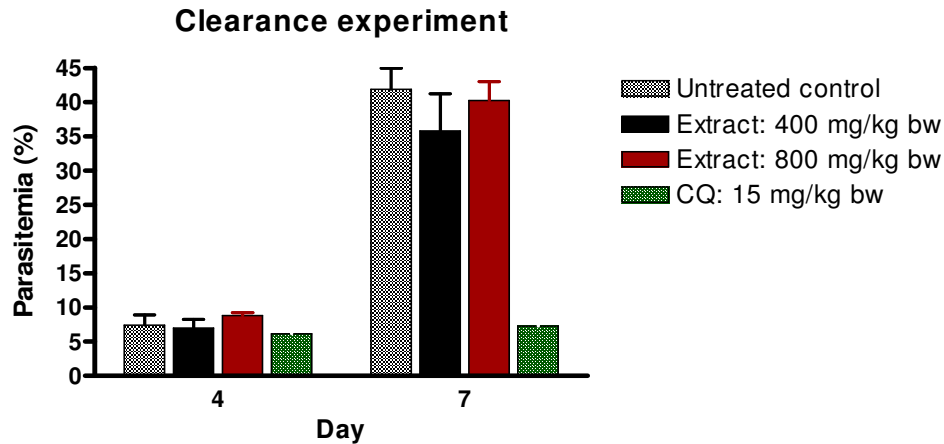


Figure 21 Clearance test: Average parasitemia on day 4 and 7 against CQR *P. yeolii* NS strain

No parasite clearance effect was observed after four days of treatment with the crude extract compared to the untreated control (Fig 21). A two-fold increase in dose did not show a dose-related response to treatment. The parasitemia of the CQ control remained unchanged at the dose treated. The steady decline in weights indicated that the disease increased in severity (Table 11).

Table 11 Averaged weights of mice in the parasite clearance test against CQR *P. yeolii* NS strain ( $\pm$ SD)

Days post-infection	Untreated control	CQ control	<i>S. aethiopicus</i> EA extract	
			400 mg/kg bw	800 mg/kg bw
0	25.21 $\pm$ 2.21	24.92 $\pm$ 1.96	24.78 $\pm$ 1.63	24.31 $\pm$ 3.24
3	24.64 $\pm$ 2.44	24.24 $\pm$ 1.99	24.23 $\pm$ 1.80	23.64 $\pm$ 2.82
7	21.79 $\pm$ 1.67	25.13 $\pm$ 2.24	21.80 $\pm$ 1.34	19.89 $\pm$ 0.85
10	19.46 $\pm$ 0.89	24.82 $\pm$ 2.68	19.69 $\pm$ 1.67	18.44 $\pm$ 1.77
12		24.08 $\pm$ 3.10	18.83 $\pm$ 1.36	17.82 $\pm$ 1.59

#### 4.1.2.2 Prophylactic test

The method for the prophylactic test was according to Chapter 7 (7.10.5). Test animals were treated for four consecutive days prior to infection and treatment was terminated on day 0 (day of infection).

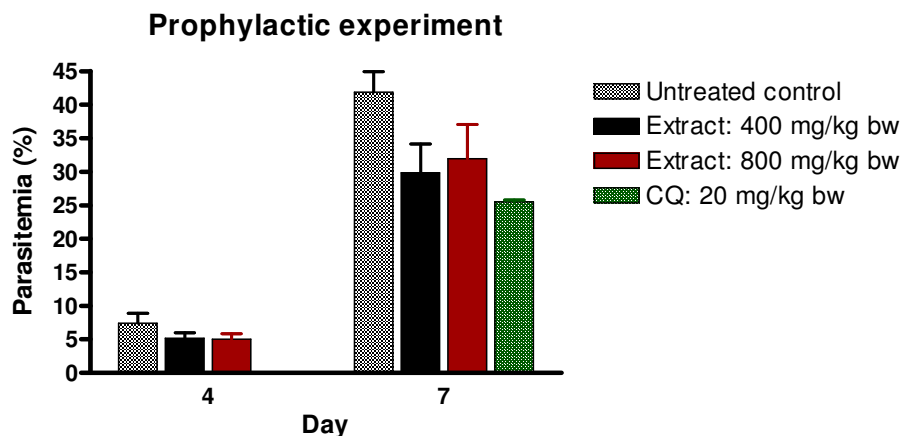


Figure 22 Prophylactic test: Average parasitemia on day 4 and 7 against CQR *P. yeolii* NS strain

Table 12 Averaged weights of mice in the prophylactic test against CQR *P. yeolii* NS strain ( $\pm$ SD)

Days post-infection	Untreated control	CQ control	<i>S. aethiopicus</i>	
			400 mg/kg bw	800 mg/kg bw
day 4 of treatment	25.39 $\pm$ 1.56	27.50 $\pm$ 0.84	27.30 $\pm$ 0.72	27.16 $\pm$ 1.27
0	25.21 $\pm$ 2.21	27.35 $\pm$ 1.19	26.50 $\pm$ 1.13	26.29 $\pm$ 1.05
3	24.64 $\pm$ 2.44	25.90 $\pm$ 1.48	25.58 $\pm$ 0.45	25.83 $\pm$ 0.97
7	21.79 $\pm$ 1.67	23.76 $\pm$ 1.22	23.19 $\pm$ 1.70	23.33 $\pm$ 1.85
10	19.46 $\pm$ 0.89	–	20.00 $\pm$ 1.16	20.50 $\pm$ 1.37
12	–	–	20.47 $\pm$ 1.37	20.80 $\pm$ 0.28

The investigation of *S. aethiopicus* for prophylactic properties did not reveal very promising results (Fig 22 and Table 12). A slight decrease in parasite growth was observed on the day 4 and 7 of treatment with the crude extract. The results were comparable for both doses tested. The CQ control showed a prophylactic effect on day 4 but a rapid increase in parasitemia was observed on day 7.

## 4.2 Bioavailability study of compound 3

A novel furanoterpenoid namely compound 3 showed promising *in vitro* activity against the malaria parasite but was not active *in vivo*. Interestingly the EA extract showed promising antiplasmodial activity in both *in vitro* and *in vivo* tests. The lack of *in vivo* efficacy of compound 3 prompted a pharmacokinetic study. Pharmacokinetic factors such as ADME can be evaluated in an *in vivo* model. Two studies were designed to understand the pharmacokinetics of compound 3 using a mouse-model. The absence of *in vivo* efficacy of compound 3 may be a result of poor absorption or metabolism to inactive compounds. The first study investigated the bioavailability of compound 3 alone or in combination as a constituent of the EA extract. Studies have shown that the route of administration and drug formulation are important parameters in accessing bioavailability and consequently *in vivo* activity (Peters et al., 2002). Therefore these parameters were investigated in this preliminary pharmacokinetic investigation. The second study investigated the *in vivo* metabolism of compound 3.

Hyphenated mass spectrometry (LC-MS/MS) based assays have largely replaced bioanalytical methods such as HPLC, gas-chromatography (GC) and GC-MS due to its rapid throughput and increased sensitivity (Shah et al., 2000). LC-MS/MS technology has become the preferred method of analyses. Parameters such as selectivity, sensitivity, recovery, accuracy and stability are imperative for method validation (Bansal et al., 2007). Several of these parameters were adhere to during method development. Most *in vivo* pharmacokinetic experiments use a large sample size which often requires that the test animals are sacrificed at each time-point. The method developed during this study evaluated bioavailability using one animal for all time-points. This eliminates intervariability between different animals and limits the amount of animals used. A washout period of 2 – 3 weeks was used to allow for the re-use of these animals in further studies.

### 4.2.1 Mass spectrometry method development

A preliminary investigation of the bioavailability of compound 3 in a mouse-model was performed using LC-MS/MS technology. An API 3200 triple quadrupole mass spectrometer (LC-MS/MS, Applied Biosystems) with ESI positive ionization with multi-reaction monitoring (MRM) was used for analyses. The mobile phase (MP) used was ACN:0.1% formic acid (6:4) with a 5  $\mu$ l infusion. The protonated molecular ion  $[M+H]^+$  at 245 (Fig 23) correlated with the high resolution MS data presented in Appendix II.

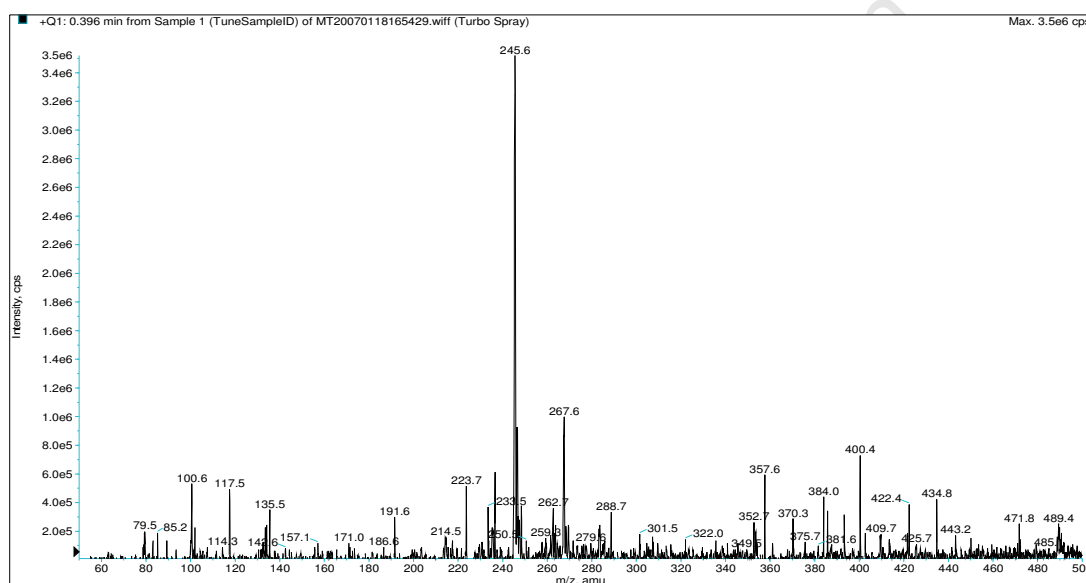


Figure 23 Mass spectrum of compound 3, molecular ion  $[M+H]^+$  at 245

The molecular ions were selected in the first mass filter and fragmented in the collision cell to form product ions (Fig 24). The most abundant product ion  $[M+H]^+$  m/z 117 was selected.

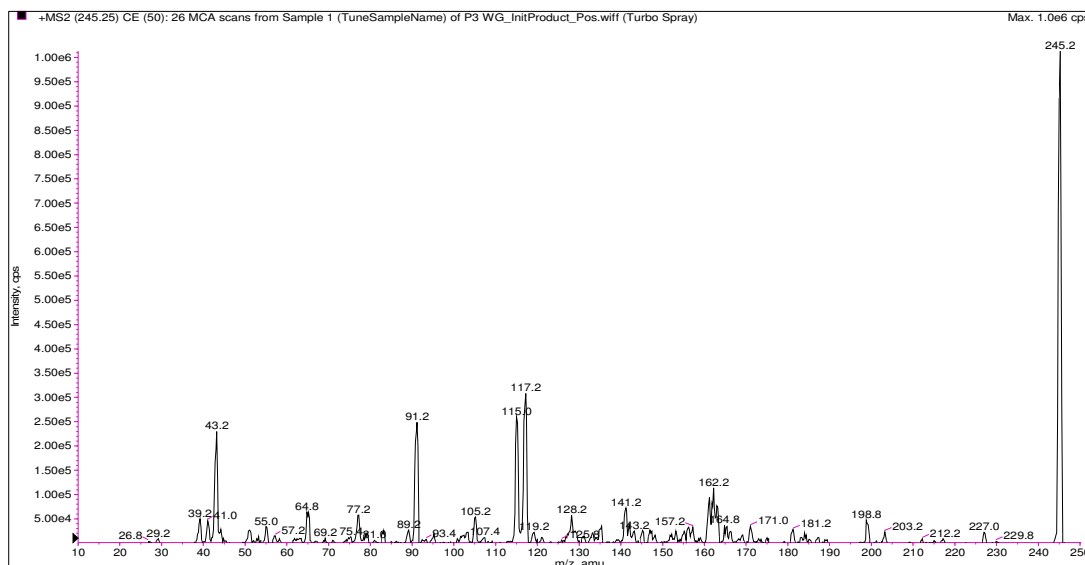


Figure 24 Product ion mass spectrum of molecular ion  $[M+H]^+$  at 245

#### 4.2.2 Optimization of mass spectrometry

A more sensitive detection method was needed to study the bioavailability of compounds. Therefore a pilot study was set-up to evaluate compound 3 extracted with a range of pH buffers and compared its absorption in different matrixes. An Applied Biosystems 3200 Q-trap mass spectrometer coupled to a high performance liquid chromatography system was used to analyse the samples. The MP used was ACN:0.1% formic acid (6:4) which was run isocratically for 1.5 min. The molecular ion of compound 3 ( $m/z$  244.2) and the major product ion of  $m/z$  116.3 were selected. Methods were according to Chapter 7 (7.11.2). Protein precipitation was unsuccessful as an extraction method. Liquid-liquid extraction was investigated as an extraction method. pH buffers ranging from pH 2 – 12 were prepared with Britton Robinson universal buffers (7.3.2). The liquid-liquid extraction used 10  $\mu$ l of spiked whole blood (10  $\mu$ g/ml) which was extracted with the respective pH buffers and EA. Extract with the pH 10 buffer was the optimal extraction method (Fig 25).



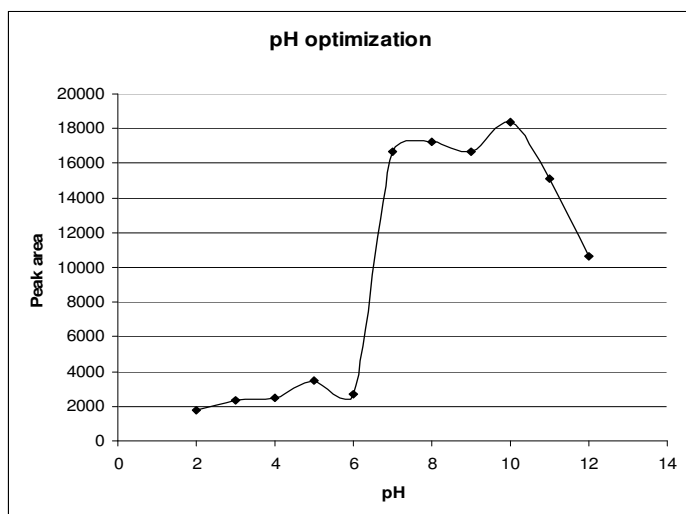


Figure 25 Extraction of compound 3 using pH buffers

The optimal absorption matrix of compound 3 as evaluated in plasma and red blood cells (RBC's). Absorption of compound 3 in plasma was increased ten-fold compared to RBC's (Table 13). Therefore plasma was used for further analysis as oppose to whole blood or RBC's.

Table 13 Percentage recovery of compound 3

Sample	% Recovery
RBC	1.93 ±0.03
Plasma	11.82 ±0.57

#### 4.2.3 Evaluation of method

A preliminary study to evaluate the sensitivity, selectivity, accuracy and reproducibility of the LC-MS/MS method was conducted. Only 10 µl of plasma was used to spike MP which was serially diluted two-fold in MP from 10 µg/ml to 0.15 µg/ml. The LC-MS/MS method was sensitive and selective to compound 3 with a lower limit of quantification (LLQ) of 0.156 µg/ml. Examples of the recovery and accuracy of compound 3 extractions are shown in Table 14 and Figure 26.

Table 14 MS data of standard (compound 3) in MP and extractions from plasma at various concentrations

Sample Name	Analyte Peak Area (counts)	Analyte Concentration (ug/mL)	Calculated Concentration (ug/mL)	Accuracy (%)
Std in MP (10 ug/ml)	1570000	N/A	No Intercept	N/A
blank	44.5	N/A	0.0234	N/A
blank	30.4	N/A	0.0226	N/A
Plasma – 10 ug/ml	155000	10	10.4	104
Plasma –10 ug/ml	143000	10	9.42	94.2
Plasma – 5 ug/ml	79700	5	4.91	98.2
Plasma – 5 ug/ml	88900	5	5.53	111
Plasma – 2.5 ug/ml	37100	2.5	2.21	88.2
Plasma – 2.5 ug/ml	44200	2.5	2.64	106
Plasma – 1.25 ug/ml	21300	1.25	1.26	101
Plasma – 1.25 ug/ml	20200	1.25	1.2	95.7
Plasma – 0.625 ug/ml	10000	0.625	0.597	95.6
Plasma – 0.625 ug/ml	10000	0.625	0.598	95.8
Plasma – 0.313 ug/ml	4940	0.313	0.304	97.2
Plasma – 0.313 ug/ml	4900	0.313	0.302	96.5
Plasma – 0.156 ug/ml	2540	0.156	0.166	106
Plasma – 0.156 ug/ml	2670	0.156	0.174	111

Std in MP = standard in mobile phase (acetonitrile:0.1% formic acid) (6:4)

N/A = not applicable

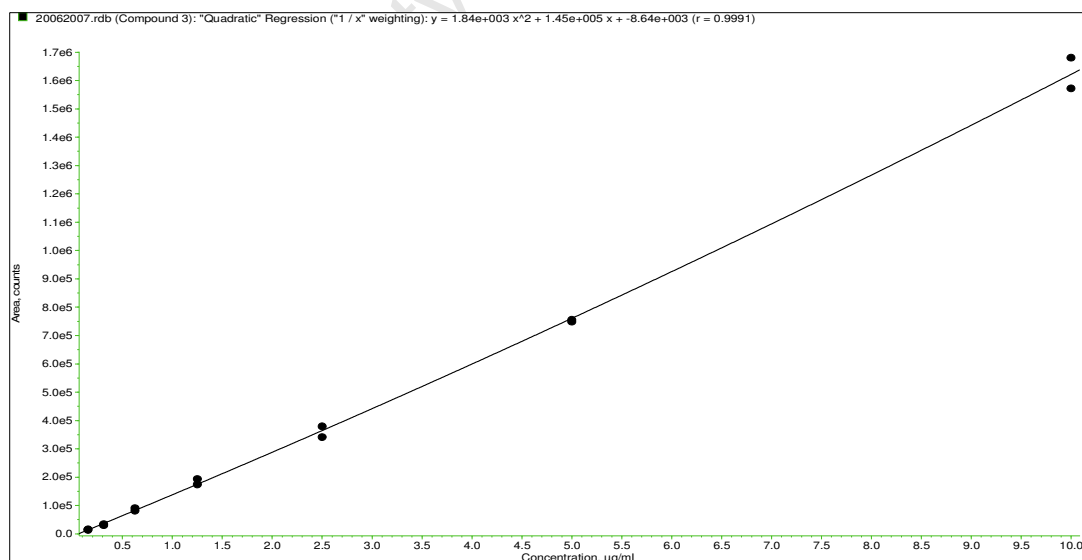


Figure 26 Standard curve of compound 3 using LC-MS/MS

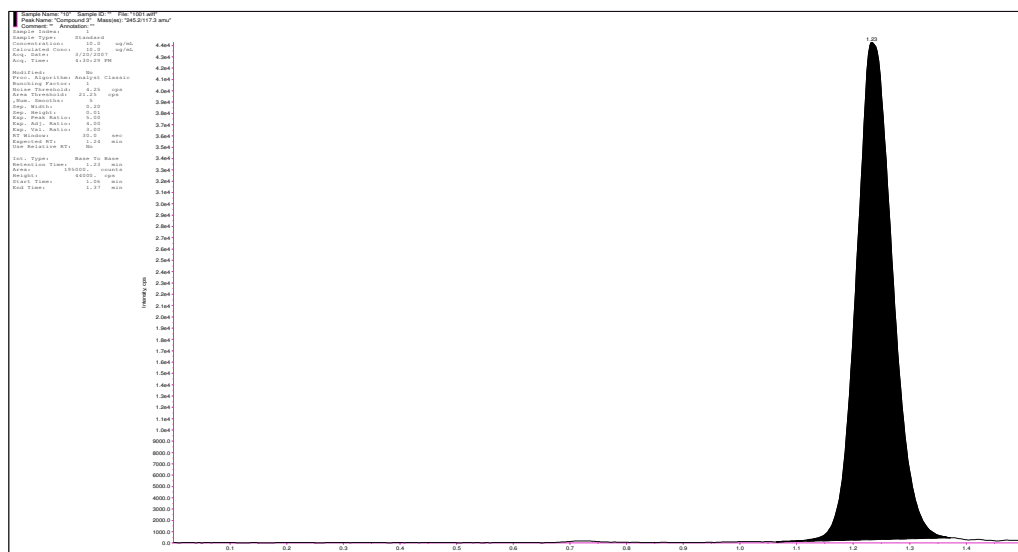


Figure 27 Chromatogram of compound 3 at the highest concentration (10 µg/ml)

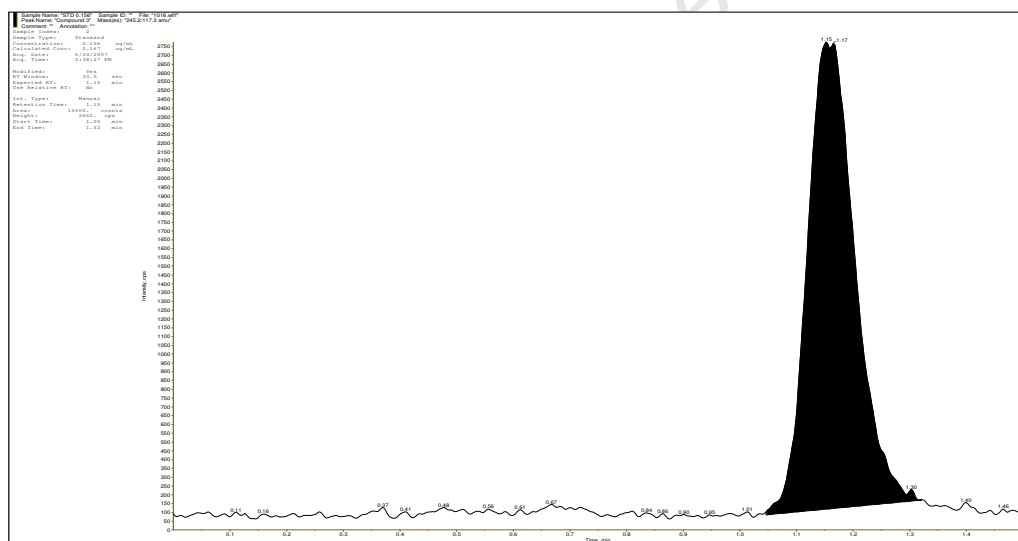


Figure 28 Chromatogram of compound 3 at the lowest concentration (0.15 µg/ml)

The method was sensitive and selective for compound 3 in plasma matrix (Fig 27 and Fig 28).

#### 4.2.4 Effect of route of administration and formulation on bioavailability

##### 4.2.4.1 Experimental conditions

An Applied Biosystems 3200 Q-trap mass spectrometer coupled to a high performance liquid chromatography system was used for analyses. The MP of ACN:0.1% formic acid (6:4) was run isocratically for 1.5 min. The molecular ion of compound 3 ( $m/z$  244.2) and the major product ion of  $m/z$  116.3 was selected in the positive ion mode, MRM. Methods were as described in Chapter 7 (7.11.3). Stock solutions of standard controls were prepared in plasma to known concentrations (10  $\mu\text{g/ml}$  to 1.5  $\mu\text{g/ml}$ ). Standard controls were run at the start and at the end of each analysis to monitor the instrument and method.

Absorption of compound 3 was evaluated by comparing the route of administration, oral vs. subcutaneous, and drug-formulation. In the first experiment compound 3 was prepared at 100 mg/kg bw in 10% DMSO and administered orally and subcutaneously. For the second experiment compound 3 was prepared at 100 mg/kg bw in a lipid-based SMEDDS formulation and administered orally and subcutaneously. The SMEDDS formulation was prepared according to Table 8. Blood samples were collected at various time intervals (Table 15).

Table 15 Time intervals of blood sampling

Interval	Time
$T_0$ = blank	Before dosing
$T_1$	30 min after dose
$T_2$	1 hr after dose
$T_3$	2 hrs after dose
$T_4$	5 hrs after dose

#### 4.2.4.2 Results

The results were captured below LLQ and were not considered accurate. However a trend in absorption was observed.

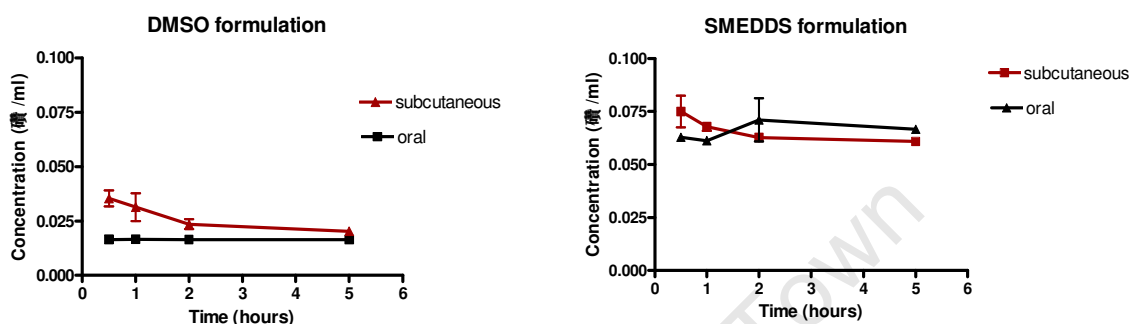


Figure 29 Relative concentrations of compound 3 dissolved in 10% DMSO or SMEDDS formulation administered either subcutaneously or orally

The results indicated an increased absorption of compound 3 in the SMEDDS formulation compared to the DMSO formulation (Fig 29). The estimated absorption concentrations at 30 min were 0.05 – 0.075 µg/ml for the SMEDDS formulation and 0.025 – 0.05 µg/ml for the DMSO formulation. The subcutaneous (SC) route of administration achieved higher concentrations after 30 min compared to oral administration. The concentration of the SC administration gradually decreased in 2 hrs and was detected for a further 3 hrs. The DMSO formulation resulted in very low levels at all time intervals. The SC administration showed greater absorption than oral administration.

### 4.3 Bioavailability of the ethyl acetate extract of *S. aethiopicus*

#### 4.3.1 Mass spectrometry conditions

An Applied Biosystems 3200 Q-trap mass spectrometer in ESI positive ionization (MRM) was used for analyses coupled to a high performance liquid chromatography system. The MP of ACN:0.1% formic acid (6:4) was run isocratically for 1.5 min. The molecular ion of compounds 1 ( $m/z$  262.1 and major product ion  $m/z$  202.3), 2 ( $m/z$  246.1 and major product ion  $m/z$  200.2) and 3 ( $m/z$  244.2 and major product ion  $m/z$  116.3) were analysed. The LC-MS spectra of compound 1 (Fig 30), compound 2 (Fig 31) and compound 3 (Fig 23 and Fig 24) correlated with the high resolution data presented in Appendix II.

Stock solutions of standard controls of compound 1, 2 and 3 were prepared in plasma to known concentrations (10  $\mu\text{g/ml}$  to 1.5  $\mu\text{g/ml}$ ). Standard controls were run at the start and at the end of each analysis to monitor the instrument and method. The EA extract was orally administered at 400 mg/kg bw and 800 mg/kg bw in the SMEDDS formulation (Table 8). Blood samples were collected at various time intervals (Table 15). Methods are described in detail in Chapter 7 (7.11.4).

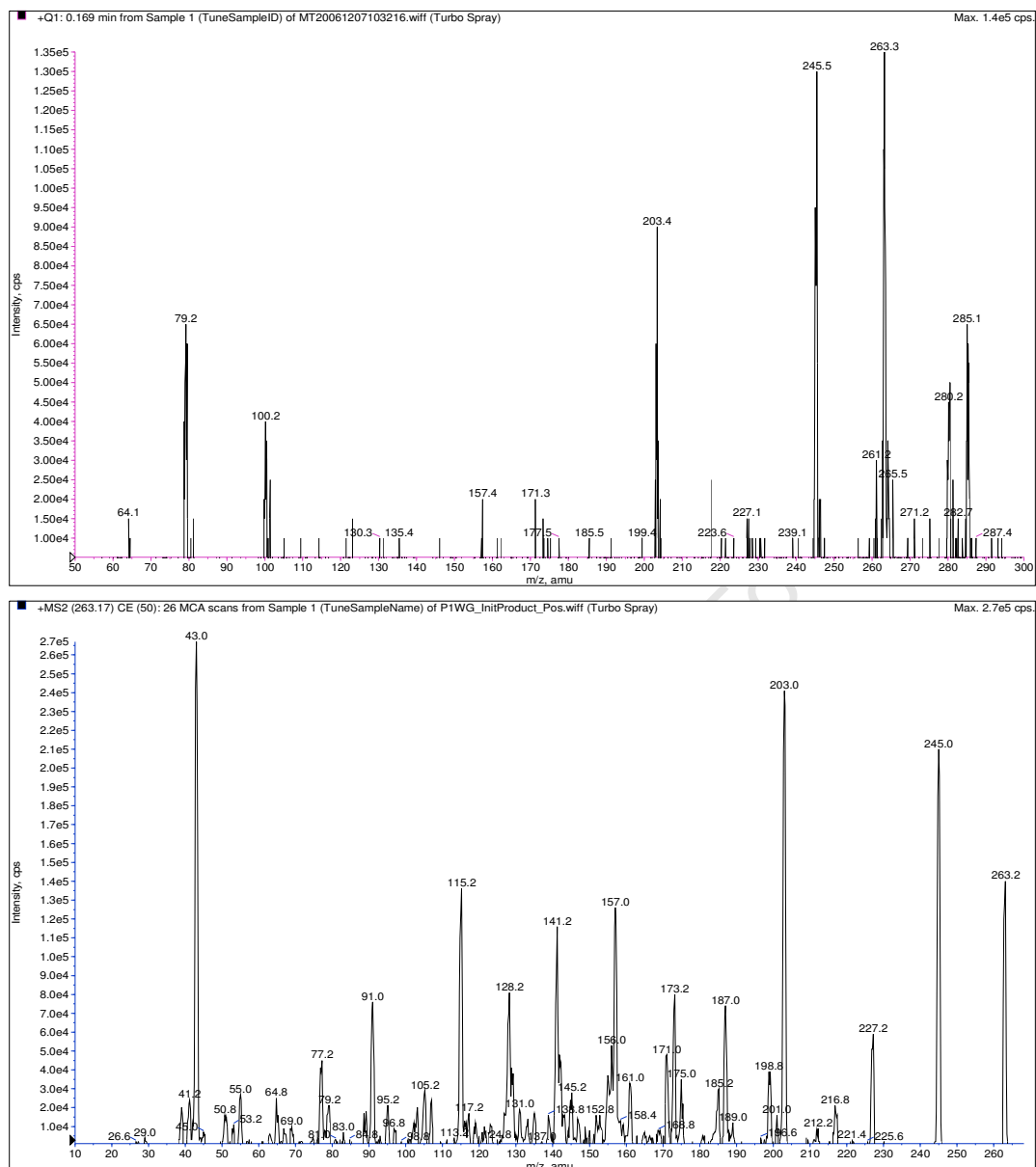


Figure 30 Mass spectrum of compound 1, molecular ion  $[M+H]^+$  at 263 and product ion mass spectrum

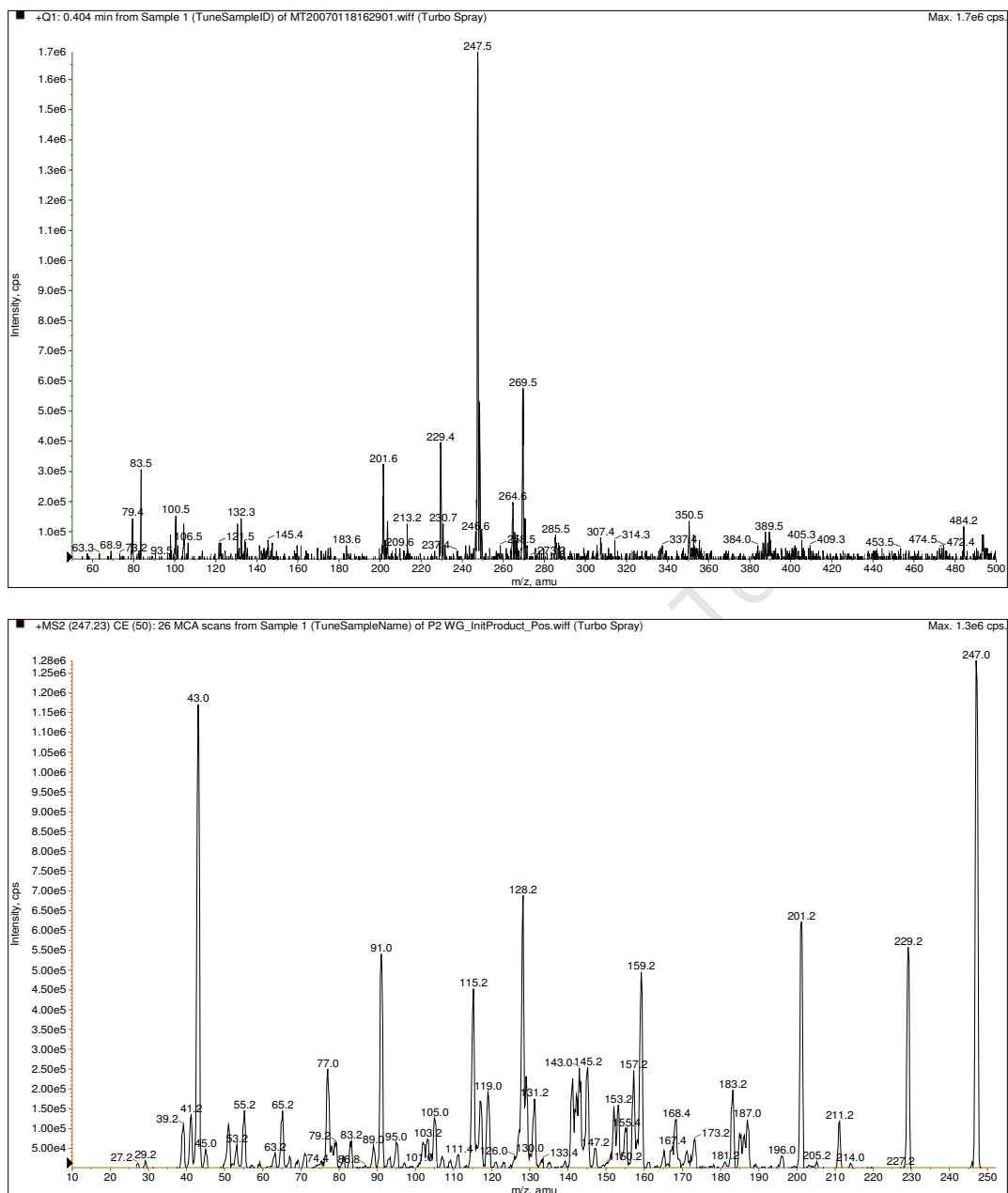


Figure 31 Mass spectrum of compound 2, molecular ion  $[M+H]^+$  at 247 and product ion mass spectrum



### 4.3.2 Results

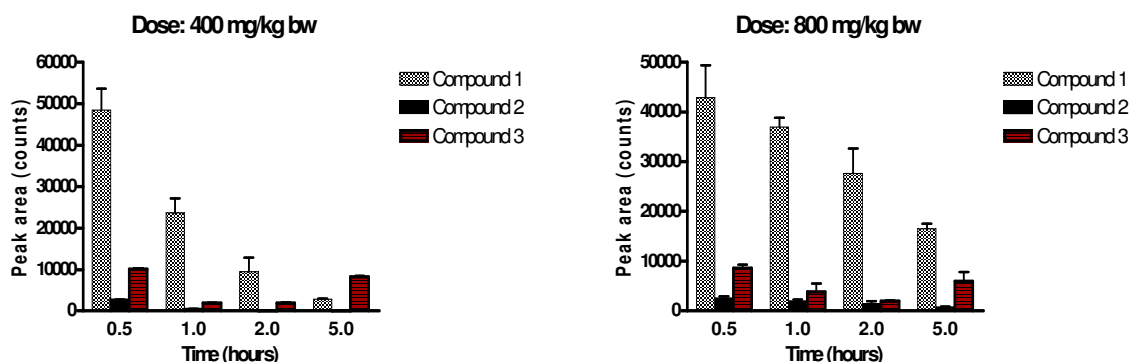


Figure 32 Detection of compounds 1 – 3 after oral administration of *S. aethiopicus* in SMEDDS formulation

Bioavailability data showed that compound 1 was absorbed in greater quantities than the other compounds at most time intervals but decreased steadily over the 5hr time period (Fig 32). Compound 1 was the major component in the EA extract (Fig 33). Compound 2 was absorbed in very low amounts. All three isolates were absorbed after 30 min. The plasma levels of compounds increased with an increase of dose. Compound 3 was detected at lower levels than compound 1 except at 5 hrs (400 mg/kg bw). Compound 3 showed a decrease in absorption from 0.5 – 2 hrs but increased again at 5hrs. The estimated concentration of compound 3 was 0.6  $\mu\text{g/ml}$  at 30 min and 5hrs.

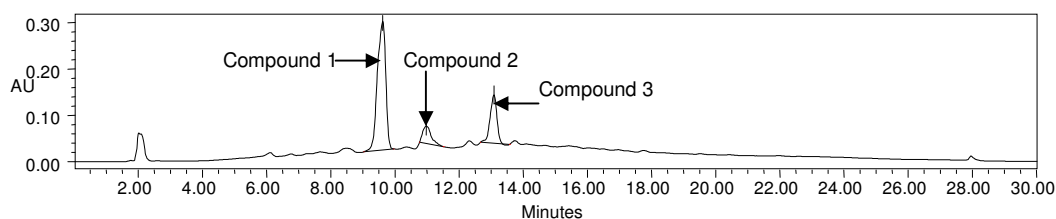


Figure 33 HPLC profile of EA extract on a C18 RP column ( $\lambda = 254 \text{ nm}$ )

Mobile phase  $\text{H}_2\text{O}:\text{ACN}$  using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

#### 4.4 Metabolism study of compound 3

The second study was designed to investigate the effect of metabolism on *in vivo* schizontocidal activity of compound 3. The purpose of this study was to detect possible metabolites in order to determine if the low plasma levels of compound 3 observed in the bioavailability study is due to metabolism. The metabolism of drugs can lead to the production of active or inactive metabolites (Peters et al., 2002). Metabolite studies attempt to create an understanding of the mechanism of action of a drug.

A systematic approach to metabolite identification and characterization can be followed. The identification of metabolites is a time-consuming and complex task requiring expertise, instrumental techniques and software applications. The first step in metabolite identification is Tandem MS experiments which includes precursor ion and neutral-loss scans (Clarke et al., 2001). These experiments are followed by LC-MS/MS experiments and accurate mass measurement which can result in the empirical formula of the novel metabolite. Common metabolite alterations can be predicted based on the structure of the drug. Thus a targeted approach for the expected metabolites can assist in the identification process. Precursor ion scans is used for the detection of novel unexpected metabolites produced by less-common metabolic pathways. Precursor ion scans can be conducted with limited knowledge of the metabolite's structure. The fragmentation pattern is used to determine if the metabolite contain unaltered fragments of the parent molecule. The bioavailability study of compound 3 prompted the next phase of the project which was to search for metabolites using precursor ion experiments.

##### 4.4.1 Mass spectrometry conditions for precursor experiment

An Applied Biosystems 3200 Q-trap mass spectrometer coupled to a high performance liquid chromatography system using a Phenomenex C<sub>18</sub> (15 cm x 4.6 mm, 5 µm) column with an injection volume of 5 µl. The MP was ACN:0.1% formic acid (6:4) and isocratic run rate of 15 min. LC-MS/MS analyses for the metabolite study were performed in the positive ion mode (MRM). The LC-MS/MS was set in the precursor ion mode and Q3 was set to stabilise the most abundant product ion of

m/z 116. A precursor ion screen scanned for metabolites with a similar fragmentation pattern as compound 3. Q1 was set to scan for large range of possible metabolites 400 to 900. The experimental conditions used for precursor ion scan analysis were according to Chapter 7 (7.11.5.1).

#### 4.4.2 Sample preparation

The activity of compound 3 was assessed in the blood stage of the parasite life-cycle. The extraction method developed for the metabolite study was not as selective as the bioavailability study. The liquid-liquid extraction method used for the bioavailability study would not extract all possible metabolites. Whole blood as opposed to plasma was used for this study in order to extract metabolites that might be present intracellularly. Urine and faeces are common elimination matrixes and were also investigated for metabolites. Extraction methods were according to methods in Chapter 7 (7.11.5.2). The test animal received 100 mg/kg bw of compound 3 in the SMEDDS formulation which was administered by gavage. The samples were collected at 30 min, 1 hr and 2 hrs time intervals. All test samples, except the blank, were pooled before being processed. This allowed for the detection of all metabolites over the two hour period. The same sample stocks were used for all LC-MS/MS analyses. Compound 3 had a retention time of 7.5 min (Fig 34).

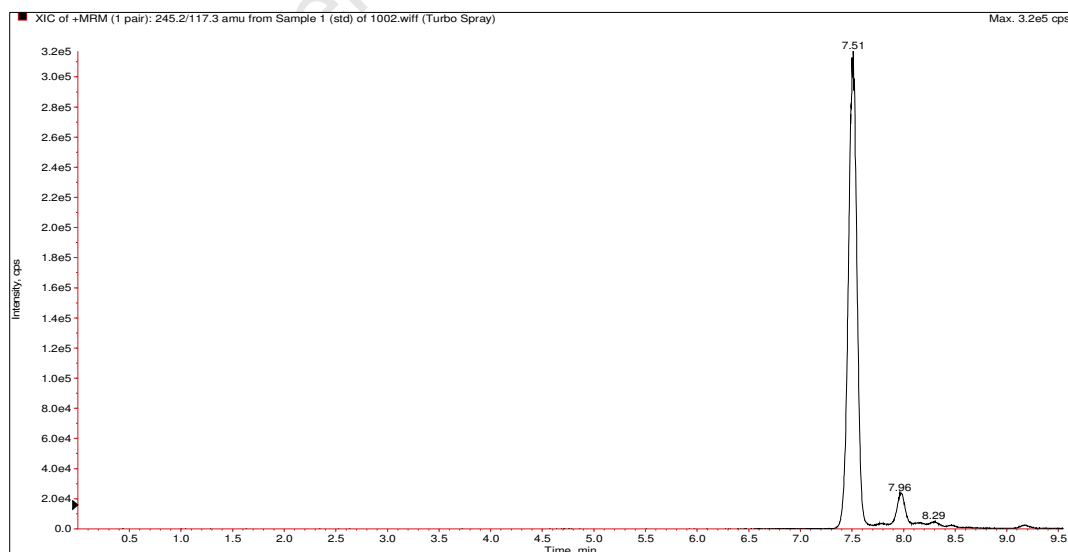


Figure 34 Chromatogram of compound 3 in MP for metabolism experiment

### 4.4.3 Results

The total ion chromatograms of the blank sample and test samples (blood, urine and faeces) were evaluated. The presence of compound 3 as well as metabolites was assessed by comparing the test sample to a blank control. Compound 3 was not present in the blood, urine or faeces and no significant differences between the test sample and the blank control were observed (Fig 35). Arrows indicate the retention time of compound 3.

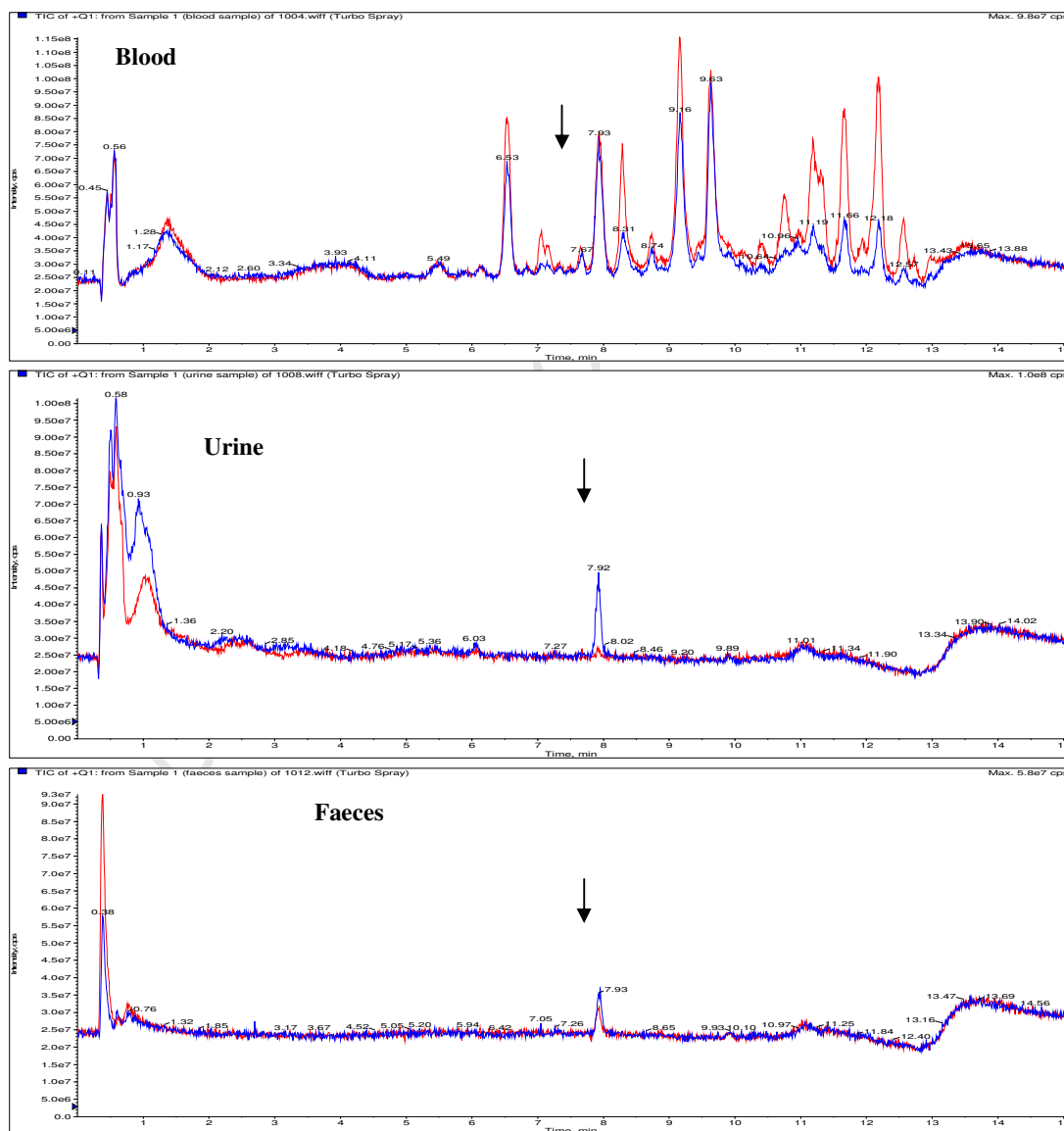


Figure 35 Comparison of total ion chromatograms for blank sample (red) to test sample (blue).

The precursor experiment was done to evaluate the presence of the product ion  $m/z$  116.3 in the test samples. Possible metabolites of compound 3 which have the same major product ion should be detected using this method. These results did not reveal any visible difference between the test samples and the blank controls (Fig 36). Arrows indicate the retention time of compound 3.

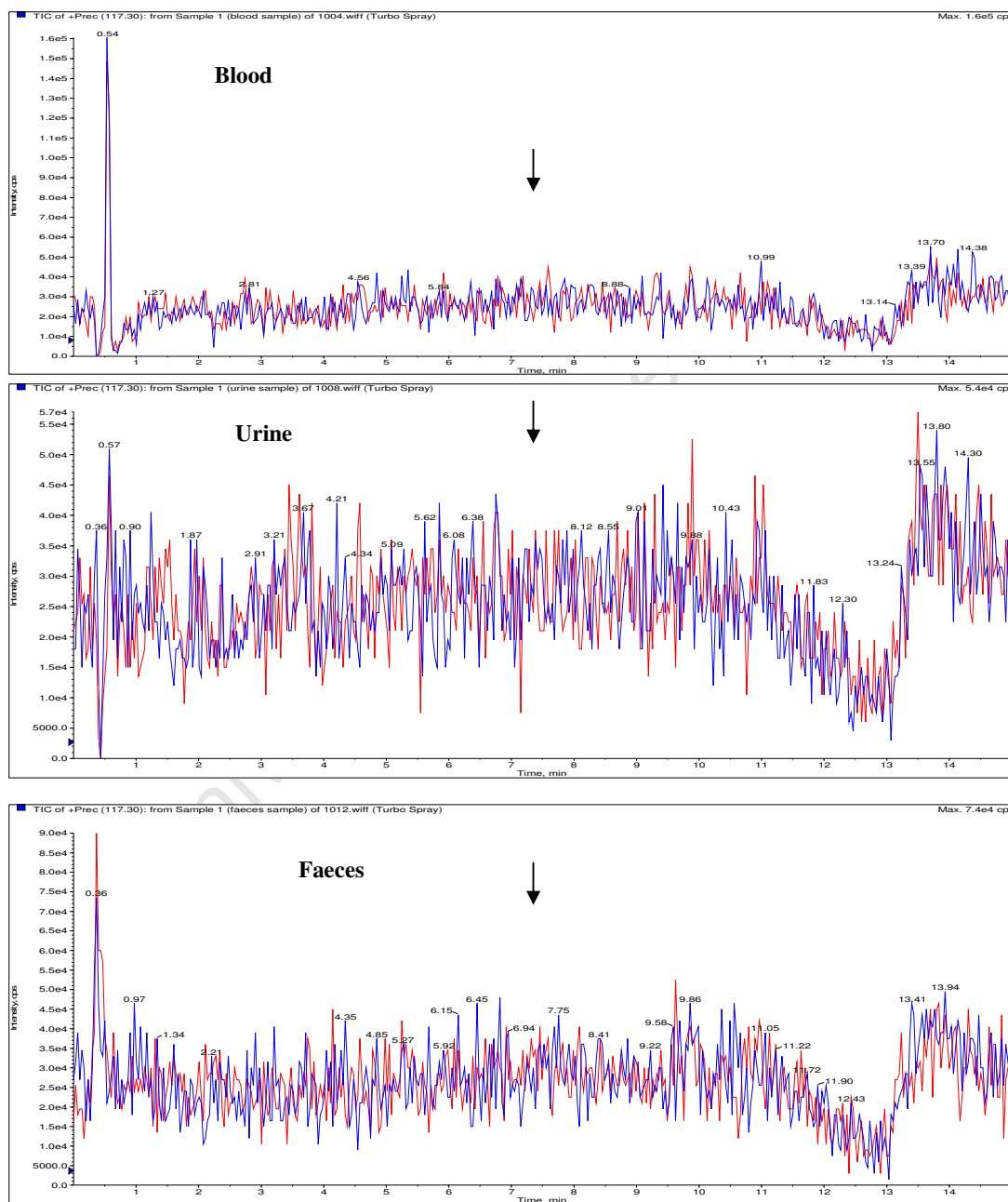


Figure 36 Comparison of total ion chromatograms for blank sample (red) to test sample (blue) in precursor experiment.

## 4.5 Discussion

### 4.5.1 Schizontocidal activity

The EA extract and compound 3 showed promising *in vitro* activity particularly against the CQR strain of *P. falciparum*. Therefore these two samples were tested for *in vivo* activity against the CQR *P. yeolli* NS strain. The EA extract inhibited parasite growth by greater than 50% on day 4 of the 4-D test. A rapid increase in parasitemia was observed after termination of treatment. Compound 3 suppressed the parasitemia (growth inhibition less than 50%) on day 4 of the 4-D test. The schizontocidal activity of the crude and compound 3 were not comparable based on the growth inhibition. These results indicated that the antiparasmodial activity of the EA extract might be due to synergism.

The schizontocidal activity of the EA extract using the 4-D suppressive test led to evaluation using a 7-D suppressive test. Two drug formulations, an aqueous-based 5% SB (with addition of 5% ethanol) and lipid-based SMEDDS formulation of the EA extract were compared at the same dose in the 7-D test. The evaluation of *in vivo* antimalarial activity by comparing drug formulations has shown to be useful in pre-clinical drug development (Peters et al., 2002). The SMEDDS formulation was inactive while the 5% SB formulation showed suppression of parasitemia on days 4 and 7. However the parasitemia remained steady from day 5 to 7 with the SMEDDS formulation. The EA extract did not have an effect on the clearance of parasitemia. A weak to moderate prophylactic effect was observed with the EA extract. Both clearance and prophylactic experiments did not show a dose-response to the high or lower dose tested.

### 4.5.2 Bioavailability and metabolism studies

The bioanalytical method developed was highly selective and sensitive for analyzing *in vivo* plasma samples. A method for the detection of compound 3 in small animals was successfully developed using LC-MS/MS technology. Two formulations of compound 3 were administered either orally or subcutaneous (SC). The lipid-based SMEDDS formulation contains a surfactant Tween 80 which has been effective in

improving bioavailability by enhancing the solubility of test compounds (Peters et al., 2002). The absorption values were below the LLQ and could not be considered accurate, but were used to show a trend. Interestingly a trend in absorption was observed with drug formulations as well as the route of administration. After 30 min the estimated absorption concentrations were 0.05 – 0.075 µg/ml for the SMEDDS formulation and 0.025 – 0.05 µg/ml for the DMSO formulation. An increase in absorption was observed with the lipid-based SMEDDS formulation compared to the 10% DMSO formulation. With both formulations the estimated concentration after 30 min was higher for the SC route than the oral administration. SC administration led to high initial absorption levels but gradually decreased in 2 hrs remaining steady at low levels for 3 hrs. A similar absorption pattern was observed for the SC administration using both formulations. Oral administration of the SMEDDS formulation showed an increased absorption after 2 hrs than for the SC administration. The oral administration of the DMSO formulation reached very low levels in blood. Compound 3 is poorly absorbed particularly when administered orally with the DMSO formulation. The plasma levels are three times less than the required *in vitro* IC<sub>50</sub>-value. The efficacy testing was performed using an oral administration of compound 3 in the DMSO formulation. Therefore poor absorption could account for its weak *in vivo* activity.

The three isolated compounds (1 – 3) of *S. aethiopicus* were evaluated in the bioavailability investigation of the EA extract. Oral administration of the EA extract at 400 mg/kg bw and 800 mg/kg bw (SMEDDS formulation) revealed that compound 1 was the most absorbed compound. HPLC data showed that compound 1 is the major peak in the crude extract. The abundance of compound 1 in the crude extract can account for the relatively high plasma levels of this compound. Dose-dependant increases in absorption levels were observed with all three compounds. Compound 1 was eliminated or metabolized within 5 hrs as evident by the steady decrease in absorption. Compound 2 was absorbed in very low amounts compared to compounds 1 and 3. Compound 3 was absorbed from the EA extract at lower levels than compound 1 except for results at 5 hrs. A trend in the absorption of compound 3 was observed at both high (800 mg/kg) and low doses (400 mg/kg) of the EA extract. This compound was eliminated or metabolized within 2 hrs but increased thereafter to

reach the same level as for the 30 min time interval. This phenomenon was also observed when the compound was orally administered in the SMEDDS formulation as a single entity. Thus compound 3 might have a delayed absorption or slow release in the SMEDDS formulation. The plasma levels of compound 3 when given as a crude extract is 10-fold higher compared to plasma levels if given alone. The increased absorption of compound 3 when given as an EA extract might explain the *in vivo* activity of the extract. However the EA extract was administered at a dose four times higher than the pure compound. The difference in concentration might account for the observed plasma levels. Studies on ART have shown that congener constituents within a plant preparation can assist in its solubility and enhance activity (Bilia et al., 2002). The combination of compounds present in the EA extract might assist in the solubility, stability and absorption, and ultimately enhance activity of compound 3.

A preliminary metabolism study of compound 3 (SMEDDS formulation) was performed by oral administration. The study period was limited to two hours. The results did not detect a significant difference between the test samples (blood, urine and faeces) and blank control. The compound might be metabolized in a shorter period of time or the metabolites can be unstable. Compound 3 showed an increase in absorption after two hours thus it is possible that an extended collection time might reveal its detection in the metabolism study. Metabolites of compound 3 should have the same fragmentation pattern. The precursor experiment was conducted to evaluate the presence of the major product ion. The expected fragmentation was not observed with this experiment. The metabolite search was limited and more intense investigation might reveal metabolites. The absence of compound 3 indicates that this compound undergoes phase I metabolism and is probably a substrate of CYP enzymes. The results indicate that the lack of *in vivo* activity of compound 3 is due to poor absorption and not rapid metabolism.



# *Chapter 5*

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*Aloe ferox*

University of Cape Town

## 5. *Aloe ferox*

### 5.1 Introduction

*A. ferox* leaves were initially collected in summer and winter (flowering season) at Kirstenbosch botanical gardens and subsequently collected at Albertinia during winter (South Africa). *A. ferox* was investigated for novel antiplasmodial compounds using the following chromatographic techniques: SPE, column chromatography, thin layer chromatography (TLC), HPLC and a chromatotron. *In vitro* and *in vivo* bioactivity testing was performed according to the methods described in Chapter 7. Additional *in vitro* tests were performed against *M. aurum* A<sup>+</sup>. The crude extract was tested for *in vivo* antiplasmodial activity using a malaria mouse-model.

### 5.2 Bioassay-guided fractionation

#### 5.2.1 Activity testing of crude extracts

The whole leaf as well as the leaf separated into the inner gel and outer leaf were extracted with different solvent systems (Table 16).

Table 16 Percentage yields of *A. ferox* extractions

Plant part	DCM	MeOH	DCM:MeOH	H <sub>2</sub> O	Leaf exudate
Whole leaf	2.07	13.21	12.78	20.34	71.8
Inner gel	3.18				
Outer leaf	1.59				

DCM = dichloromethane, MeOH = methanol, EA = ethyl acetate, DCM:MeOH (1:1)

The crude extracts were tested for *in vitro* antiplasmodial activity against the CQS D10 strain of *P. falciparum*. The dichloromethane (DCM) extraction of the whole leaf was shown to be the most active extract (Table 17).

Table 17 Percentage parasite survival of *A. ferox* whole leaf extracts using a 3-point concentration test against CQS D10 strain of *P. falciparum* (n = 1)

Extraction solvent	%	Parasite	Survival
	25 µg/ml	12.5 µg/ml	6.25 µg/ml
DCM	16.99	40.00	60.95
MeOH	88.93	100.74	105.08
DCM:MeOH	97.34	91.55	109.70
H <sub>2</sub> O <sup>a</sup>	103.24	92.39	88.02
Leaf exudate	104.34	99.12	85.02
	<b>30 ng/ml</b>	<b>15 ng/ml</b>	<b>7.5 ng/ml</b>
Chloroquine	39.25	42.62	66.84

n = number of experiments in duplicate

Seasonal variation in IC<sub>50</sub>-values was observed for leaves collected at Kirstenbosch botanical gardens (Fig 37). Leaves collected in winter were more active than the summer collections.

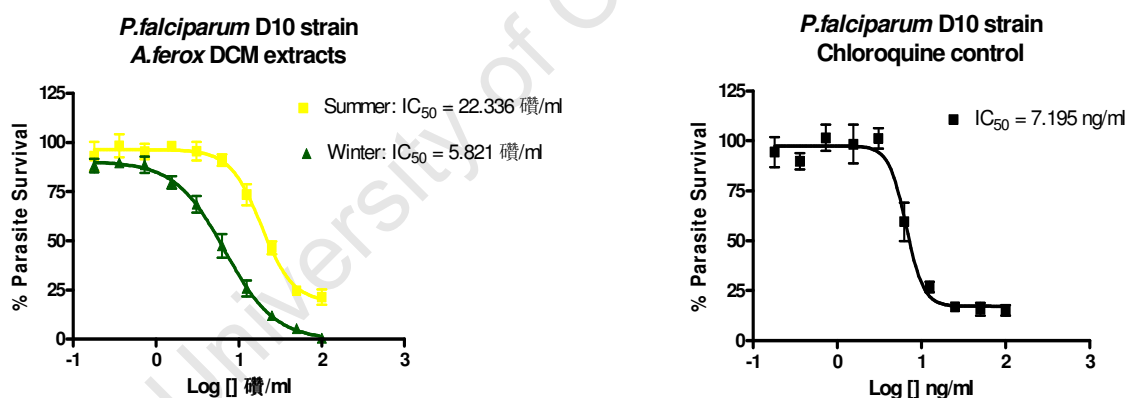


Figure 37 Pooled dose-response curves of whole leaf extracts (n = 3) and CQ control (n = 2) against CQS D10 strain of *P. falciparum*

Winter collections were preferred based on the observed increase in activity. The HPLC profiles of collections in winter at two different locations namely Kirstenbosch botanical gardens and Albertinia were compared.

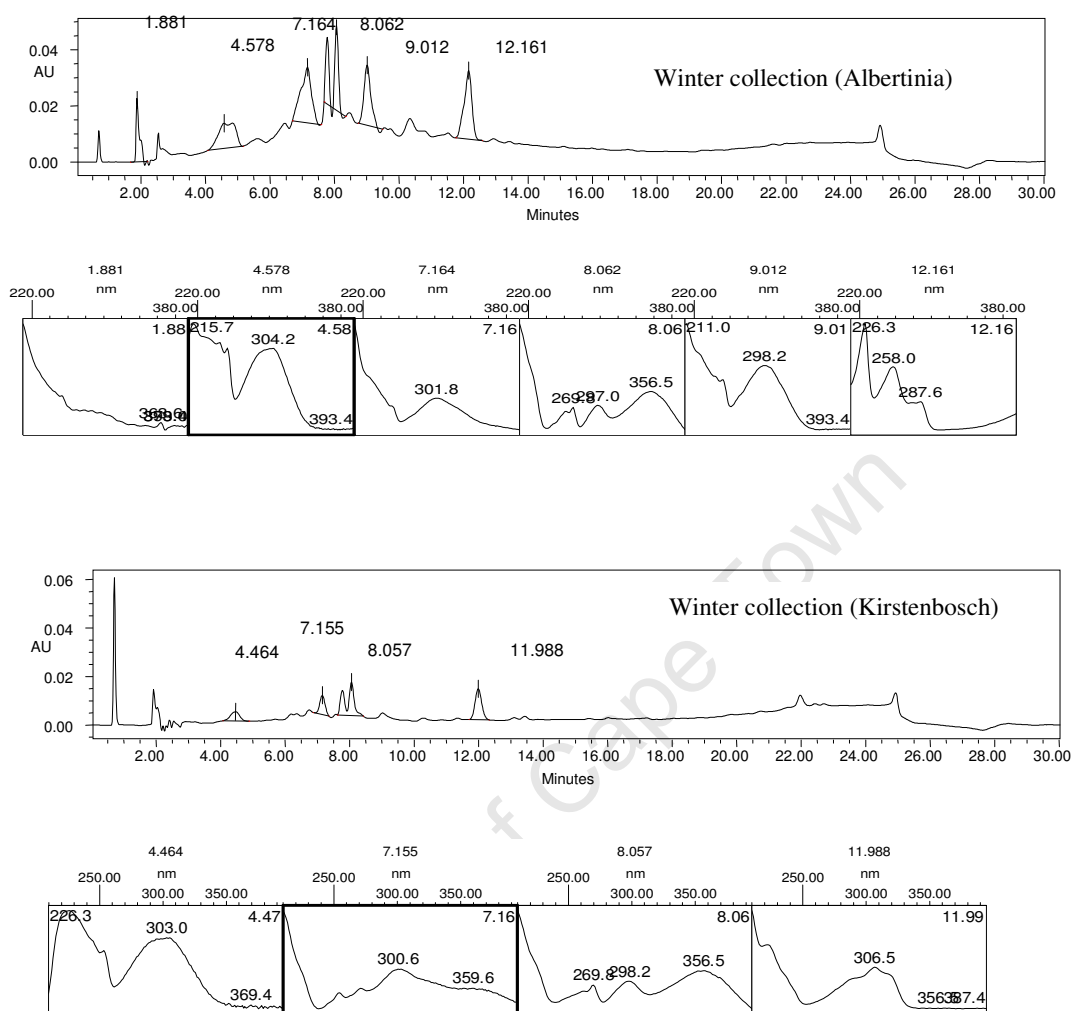


Figure 38 HPLC profiles and UV spectrum of *A. ferox* whole leaf DCM extracts on a C18 RP column ( $\lambda = 300$  nm)

HPLC conditions: Injection: 50  $\mu$ l injection of a 1mg/ml solution. Mobile phase ACN:H<sub>2</sub>O using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

The HPLC profiles of plant extracts from two different locations compared well with each other (Fig 38). Several peaks from the Kirstenbosch collection appeared smaller when compared to the Albertinia collection indicating that the compound/s are in lesser amounts. The peak at retention time 12.1 min does not appear in the Kirstenbosch collection. A peak at retention time 11.9 min appears in the

Kirstenbosch collection but not in the Albertinia collections. The UV spectral data of the peak at 8.1 min observed in both collections corresponds with spectra for aloin-like compounds. Aloin type spectra reveal major bands at approximately 270 nm, 300 nm and 360 nm (Zonta et al., 1995). Aloin (Sigma) was analyzed using HPLC under the same conditions as the crude extracts (Fig 39). The HPLC analyses showed that there are discrepancies between collections at the two locations.

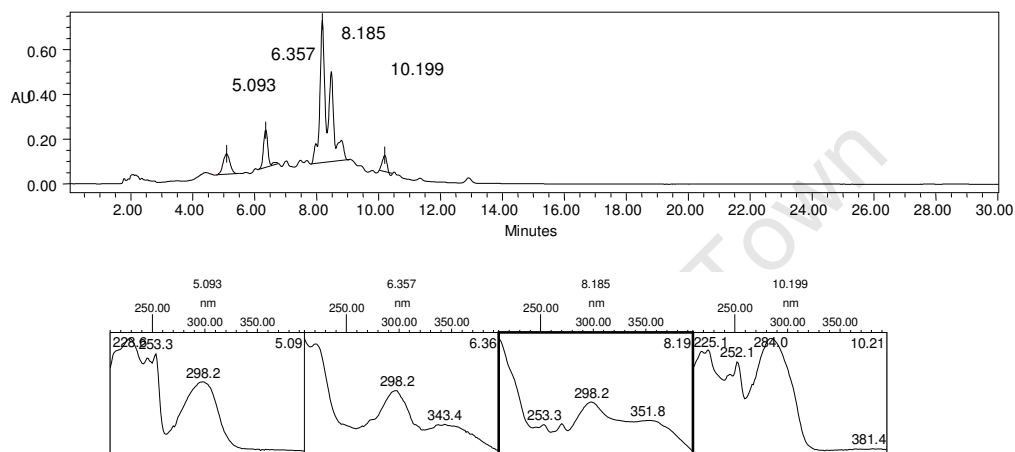


Figure 39 HPLC profiles and UV spectrum of aloin on a C18 RP column ( $\lambda = 300$  nm)

HPLC conditions: Prepared to 1 mg/ml in DMSO. Mobile phase  $H_2O:ACN$  using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

The extracts were tested for *in vitro* antiparasmodial activity and cytotoxicity (Table 18). The whole leaf DCM extract was tested against the CQR K1 strain of *P. falciparum*. The results revealed an averaged  $IC_{50}$ -value of 47.3  $\mu g/ml$  against the CQR K1 strain of *P. falciparum*. The antiparasmodial activity of the gel was comparable against both strains of the parasite. The outer leaf extract collected at Kirstenbosch was more active against the CQR K1 strain than the Albertinia collections. The extracts did not show any cytotoxicity against the Chinese Hamster Ovarian (CHO) cell-line.

Table 18 IC<sub>50</sub>-values of *A. ferox* DCM extracts against CQS D10 strain of *P. falciparum*

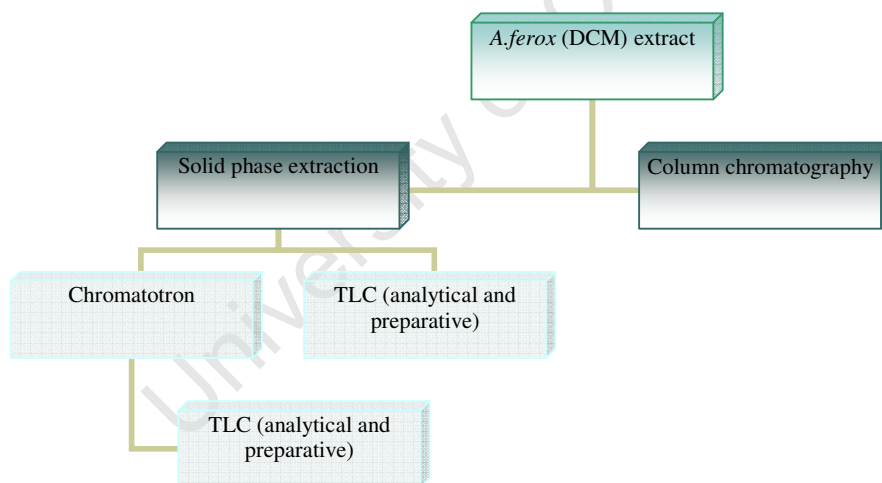
Plant part	Location	Antiplasmodial		Cytotoxicity	RI <sup>a</sup>
		D10: IC <sub>50</sub> (µg/ml)	K1: IC <sub>50</sub> (µg/ml)	CHO: IC <sub>50</sub> (µg/ml)	
Whole leaf	Kirstenbosch	–	47.30 ±6.67	>100	–
Gel	Kirstenbosch	20.27 ±2.95	25.30 ±2.97	> 100	1.25
Outer leaf	Kirstenbosch	83.8 ±10.86	62.81 <sup>b</sup>	> 100	0.75
Gel	Albertinia	28.87 ±3.07	35.55 ±0.07	> 100	1.23
Outer leaf	Albertinia	>100	>100	>100	
Chloroquine		16.77 ±0.82	269.61 ±113.1	–	–
Emetine		–	–	0.06	–

IC<sub>50</sub>-values are given as a mean value with standard deviations of 2 or 3 independent experiments

Resistance index (RI<sup>a</sup>) = K1 IC<sub>50</sub>/D10 IC<sub>50</sub>

b = tested on one occasion in duplicate

Several chromatographic techniques were used to fractionate and purify compounds from DCM extracts of *A. ferox* as summarized in Scheme 3.



Scheme 3 Fractionation procedure of *A. ferox* extract (TLC = thin layer chromatography)

### 5.2.2 Compound 1

The whole leaf DCM extract was fractionated using SPE (7.4). Activity testing using a three-point concentration test revealed that SPE fractions of 60%, 80% and 100% ACN are the most active (Table 19). Percentage survival is given as a mean value of two independent experiments performed in duplicate ( $\pm$  SD)

Table 19 Percentage parasite survival of *A. ferox* SPE fractions using a 3-point concentration test against CQS D10 strain of *P. falciparum*

SPE fractions	%	Parasite	Survival
	25 $\mu$ g/ml	12.5 $\mu$ g/ml	6.25 $\mu$ g/ml
100% A C N	2.91 $\pm$ 4.12	7.20 $\pm$ 1.35	14.86 $\pm$ 3.75
80% A C N	5.20 $\pm$ 3.48	8.15 $\pm$ 4.02	23.07 $\pm$ 9.12
60% A C N	4.53 $\pm$ 2.94	20.65 $\pm$ 3.40	27.98 $\pm$ 4.13
40% A C N <sup>a</sup>	21.85	40.83	50.66
20% A C N	73.80 $\pm$ 13.41	77.52 $\pm$ 9.72	75.28 $\pm$ 11.02
	30 ng/ml	15 ng/ml	7.5 ng/ml
CQ	30.60 $\pm$ 9.54	42.56 $\pm$ 13.6	68.13 $\pm$ 14.18

a = tested once in duplicate

SPE was optimized from 20% ACN increments to 5% increments which revealed that fractions at 55%, 80%, 85% and 90% ACN were the most active (Fig 40).

SPE fractions against *P. falciparum* D10 strain

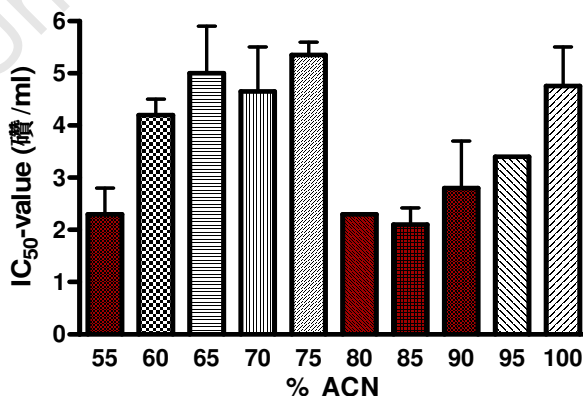


Figure 40 Average IC<sub>50</sub>-values of SPE fractions against CQS D10 strain of *P. falciparum*

Table 20 SPE fractions separated using TLC

SPE Fraction	PTLC band	Number of TLC Bands	Rf-value
100% ACN	3	3	0.265, 0.296, 0.857
	4	2	0.298, 0.309
	5	1	0.301
	6	2	0.256, 0.280
	8	4	0.269, 0.289, 0.404, 0.904
	12	8	0.250, 0.289, 0.519, 0.558, 0.635, 0.692, 0.731, 0.885
	15	4	0.278, 0.463, 0.556, 0.667
90% ACN	17	3	0.260, 0.620, 0.780
	2	3	0.265, 0.286, 0.857
	3	1	0.277
	7	3	0.278, 0.704, 0.926
	8	2	0.220, 0.780
85% ACN	9	2	0.220, 0.840
	2	2	0.224, 0.857
	3	1	0.286
	4	1	0.265
	6	3	0.269, 0.596, 0.885
	8	2	0.278, 0.926
	10	2	0.220, 0.800
80% ACN	11	2	0.220, 0.800
	2	3	0.276, 0.857, 0.979
	3	1	0.286
	4	1	0.256
	5	3	0.250, 0.596, 0.885
	6	2	0.220, 0.780
	7	2	0.220, 0.820
65% ACN	1	2	0.245, 0.878
	2	1	0.298
	3	2	0.256, 0.878
	4	4	0.276, 0.575, 0.701, 0.759
60% ACN	1	2	0.245, 0.878
	2	1	0.277
	3	1	0.247
	4	4	0.299, 0.598, 0.713, 0.736
55% ACN	1	2	0.296, 0.420
	2	3	0.269, 0.423, 0.885
	3	2	0.220, 0.720
	4	4	0.264, 0.575, 0.689, 0.747
50% ACN	1	4	0.209, 0.235, 0.259, 0.321
	2	3	0.269, 0.442, 0.904
	3	3	0.264, 0.586, 0.759

Mobile phase = EA:Tol:AA (4:16:1)



TLC separations of SPE fractions led to the isolation of compound 1 which was obtained by combining TLC bands with R<sub>f</sub>-values of 0.3 (Table 20). Compound 1 was not detected using HPLC with UV detection therefore it was evaluated using LC-MS technology. Conditions for LC-MS analysis are described in Chapter 7 (7.11). The LC-MS was scanned for possible mass ions from m/z 50 to 500 with a cycle time of 0.5 seconds. Compound 1 was infused using a mobile phase (MP) of ACN:0.1% formic acid (1:1). LC-MS data revealed that the mass ion of m/z 403.3 with negative ionization might be the molecular ion (Fig 41). The chemical structure of this compound could not be determined using NMR due to insufficient material.

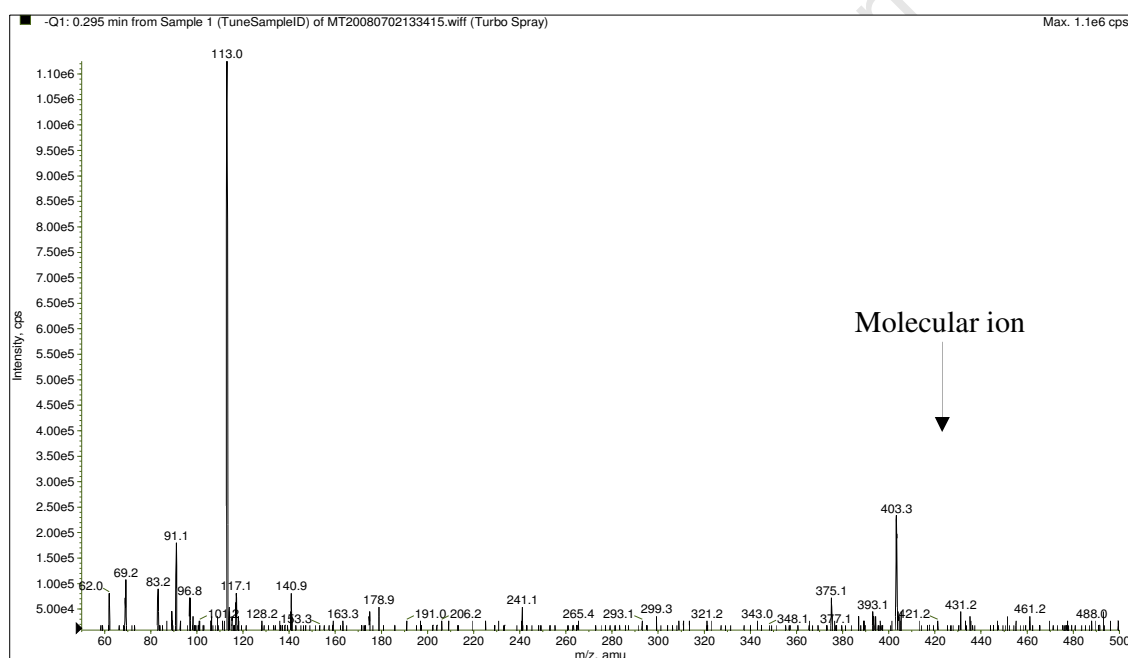


Figure 41 LC-MS spectrum of compound 1.

Negative ionization in MP ACN:0.1% formic acid (1:1), infused with 15  $\mu$ l.

### 5.2.3 Compound 2

SPE fractions obtained from the whole leaf DCM extract were also separated using a chromatotron (7.4.1). The chromatotron works on the same separation principle as TLC. This procedure is not as time consuming as TLC and can be used as a preparative method of separation. Therefore the chromatotron was used to optimize the fractionation process as an alternative to TLC. However, fractionation using the chromatotron method resulted in high yields but poor separation and irreproducibility (Table 21). Chromatotron fractions were further purified using preparative thin layer chromatography (PTLC) and analytical TLC techniques. The 65% ACN SPE fraction was not compatible with the chromatotron method and was separated only using PTLC.

Table 21 SPE fractions separated using a chromatotron

SPE fraction	Chromatotron fraction	Mobile phase	% Yield
60% ACN	A	Hex:EA (8:2)	15.19
	B	MeOH:EA (1:1)	53.21
80% ACN	A	Hex:EA (8:2)	25.29
	B	MeOH:EA (1:1)	15.99
	C	MeOH	7.99
	D	MeOH:H <sub>2</sub> O	3.64
	E	MeOH wash	12.22
85% ACN	A	Hex:EA (8:2)	2.26
	B	Hex:EA (8:2)	7.22
	C	MeOH:EA (1:1)	5.89
	D	MeOH:EA (1:1)	16.59
	E	MeOH	14.42
	F	MeOH:H <sub>2</sub> O	10.61
90% ACN	A	Hex:EA (8:2)	6.73
	B	Hex:EA (8:2)	45.56
	C	MeOH:EA (1:1)	10.04
	D	Between bands	36.21
	E	Waste	2.05
	F	MeOH	1.28

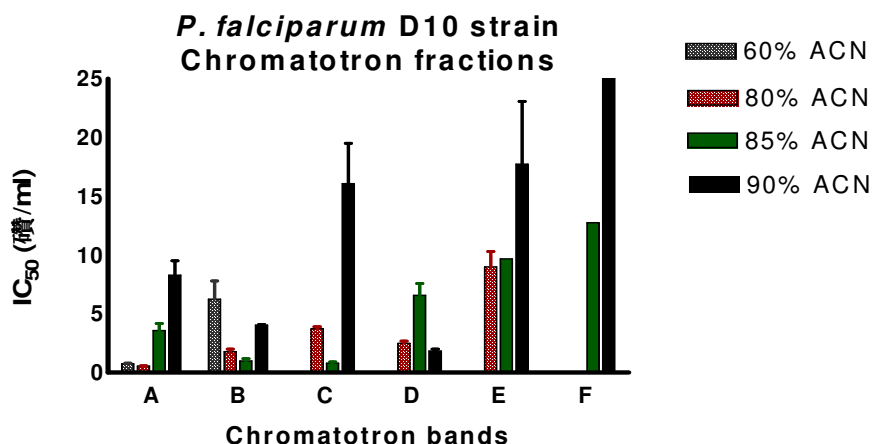


Figure 42 Averaged  $IC_{50}$ -values of chromatotron fractions obtained from SPE fractions against the CQS D10 strain of *P. falciparum* ( $n = 2$ )

An enhancement in activity was observed with fractions obtained from the chromatotron (Fig 42). The results showed that for chromatotron band A, the 60% and 80% ACN SPE fractions were the most active. Chromatotron band B was the most active for the 80% and 85% ACN SPE fractions. Chromatotron band C of the 85% ACN SPE fraction showed the most activity. Activity was observed with chromatotron band D of the 80% and 90% ACN SPE fractions. Chromatotron bands E and F did not show an increase in activity with  $IC_{50}$ -values higher than 10.

Further fractionation of active chromatotron bands was performed on PTLC and subjected to further activity testing. PTLC bands obtained from chromatotron band A of the 80% ACN SPE fraction and band D of the 90% ACN SPE fraction showed activity in an initial three-point concentration test (Table 22). Percentage survival is given as a mean value of two independent experiments ( $\pm$  SD).

Table 22 Percentage parasite survival of PTLC bands using a 3-point concentration test against the CQS D10 strain of *P. falciparum*

Chromatotron fraction	PTLC band	%	Parasite	Survival
		10 µg/ml	5 µg/ml	2.5 µg/ml
85% ACN SPE, band B	1	96.86 ±1.19	98.05 ±6.43	105.65 ±11.53
	2	97.20 ±14.57	91.30 ±4.67	94.65 ±1.48
	3	95.35 ±2.90	96.55 ±0.07	81.45 ±12.94
90% ACN SPE, band D	1	16.60 ±17.68	26.40 ±17.54	44.75 ±16.33
	2	89.15 ±3.32	93.90 ±0.99	84.90 ±0.28
	3	39.70 ±23.76	67.50 ±10.61	76.25 ±11.67
80% ACN SPE, band A	4	29.70 ±14.57	63.20 ±3.68	81.90 ±9.19
	1	8.75 ±8.27	19.35 ±11.67	34.90 ±21.21
	2	80.40 ±9.90	88.60 ±19.80	87.10 ±11.74
	3	29.30 ±17.39	52.45 ±12.66	73.15 ±5.73
	4	97.60 ±15.56	93.05 ±11.24	93.70 ±11.60
85% ACN SPE, band C	1	87.45 ±6.01	95.90 ±7.35	88.15 ±4.88
	2	96.10 ±2.69	105.10 ±1.56	93.10 ±4.24
60% ACN SPE, band A	1	74.70 ±7.64	97.35 ±0.07	96.95 ±3.61
	2	104.75 ±2.05	95.50 ±0.57	96.35 ±3.46
		30 ng/ml	15 ng/ml	7.5 ng/ml
Chloroquine		30.55 ±10.96	64.30 ±7.78	90.75 ±15.34

Mobile phase = Toluene: Ethyl acetate (93:7)

The PTLC bands which showed activity in the three-point concentration test were tested in a full dose-response assay to determine their IC<sub>50</sub>-values (Table 23). Band 1 of both chromatotron fractions was the most active.

Table 23 IC<sub>50</sub>-values of active PTLC bands against the CQS D10 strain of *P. falciparum*

Chromatotron fraction	PTLC band	IC <sub>50</sub> -value (µg/ml)
80% ACN SPE, band A	Band 1	1.30 ±0.28
	Band 3	8.30 ±0.28
90% ACN SPE, band D	Band 1	2.60 ±0.42
	Band 3	10.00 ±1.56
	Band 5	7.30 ±1.70

Mobile phase = Toluene: Ethyl acetate (93:7)

IC<sub>50</sub>-values are given as a mean value (± SD) of 2 independent experiments

Table 24 Chromatotron fractions separated via TLC

Chromatotron fraction	PTLC band	Number of TLC bands	Rf-value
80% ACN SPE, A	Band 1	2	0.071, 0.476
	Band 3	3	0.042, 0.333, 0.370
90% ACN SPE, D	Band 1	2	0.055, 0.436
	Band 3	3	0.036, 0.333, 0.458
	Band 5	4	0.071, 0.220, 0.488, 0.625

Analytical TLC was used to purify active chromatotron fractions (Table 24). Compound 2 was isolated from chromatotron band D of the 90% ACN SPE fraction using TLC (Rf-value = 0.22). A band with a RF-value of 0.22 was also found in several active SPE fractions separated via TLC (Table 20). This gave an indication that this common band might be the compound responsible for the bioactivity. The purity of compound 2 was evaluated using HPLC.

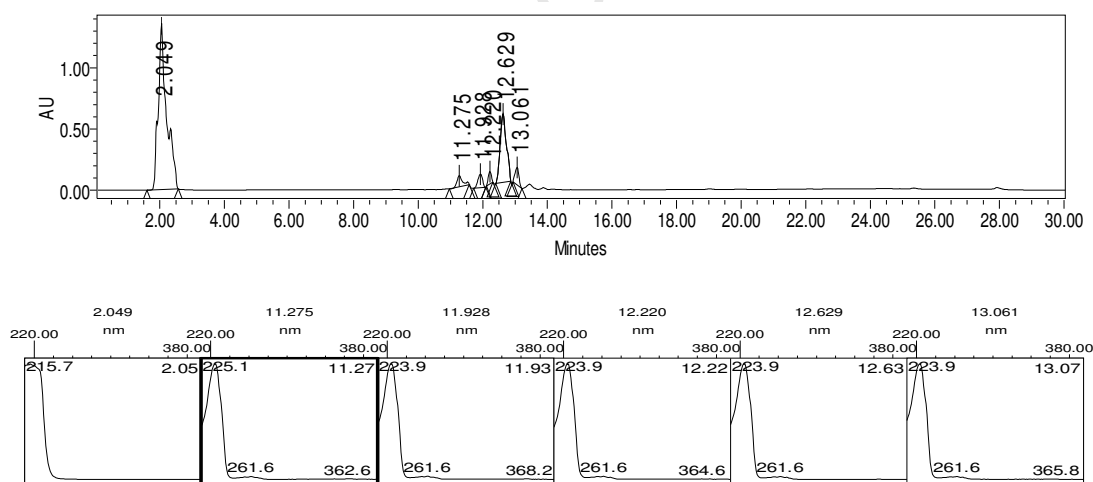


Figure 43 HPLC profile and UV spectra of compound 2 on a C18 RP column ( $\lambda = 230$  nm)

HPLC conditions: Mobile phase H<sub>2</sub>O:ACN using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

HPLC analysis of compound 2 revealed that the compound might have decomposed as indicated by the smaller peaks with the same absorbance as the major peak (Fig

43). The UV-spectrum of compound 2 revealed absorbance around 223.9 nm, 261.6 nm and 364.6 nm. The absorbance data suggests compound 2 has a chromone-like structure (Zonta et al., 1995). LC-MS and NMR analyses could not be performed due to instability problems.

### 5.2.4 Compounds 3 and 4

The inner gel was shown to be the plant part consisting of the most active constituents (van Wyk et al., 1996). The extractions of the inner gel and outer leaf were not very active against the parasite (Table 18).

Table 25 PTLC bands of *A. ferox* gel and outer leaf extracts

Plant part	PTLC band	IC <sub>50</sub> (µg/ml)
Gel	1	13.75 ±1.91
	2	6.80 ±0.71
	3	25.25 ±0.92
	4	26.30 ±ND
	5	21.14 ±ND
	6	>100 ±ND
Outer leaf	1	8.65 ±1.20
	2	11.45 ±3.89
	3	13.40 ±1.56
	4	17.55 ±1.77
	5	8.75 ±0.35
	6	13.35 ±4.74
	7	2.85 ±0.21
	8	6.25 ±0.92
	9	6.90 ±2.40
CQ (ng/ml)		19.68 ±ND

ND = Not determined

Mobile phase = Toluene: Ethyl acetate: Acetic acid (16:4:1)

IC<sub>50</sub>-values are given as a mean value with ± standard deviations of 2 independent experiments

Fractionation showed an enhancement in activity of the whole leaf extract (Table 25). TLC band 2 of the gel extract was the most active gel fraction. Interestingly, the inactive outer leaf extract revealed several active fractions when separated. TLC bands 1, 5, 7, 8 and 9 were the most active outer leaf fractions. The outer leaf extract

was investigated further due to the promising results obtained during fractionation as well as sufficient amounts as the gel largely consists of water. TLC separation resulted in low yields, thus column chromatography (CC) was investigated, as an alternative method, as described in Chapter 7 (7.4.1). The outer leaf DCM extract was fractionated using CC (Table 26). Fractions were analyzed by TLC and those with similar profiles were combined.

Table 26 Fractions collected via column chromatography

Fractions	Mobile phase Ethyl acetate:Petroleum ether
1 – 48	7:3
49 – 55	4:6

The purity of combined fractions was analyzed via HPLC. Two peaks detected at retention times 8.4 min and 12.1 min were dominant in most CC-fractions.

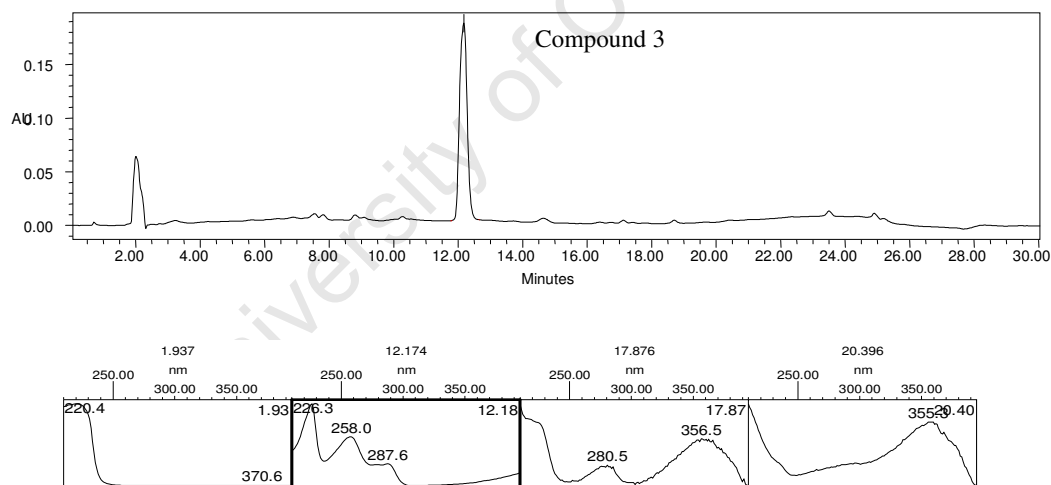


Figure 44 HPLC profile of compound 3 on a C18 RP column ( $\lambda = 250$  nm)

HPLC conditions: Mobile phase H<sub>2</sub>O:ACN using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min)

Compound 3 was obtained by combining CC-fractions 17 – 19, 25 – 27 and 28 – 29 which revealed a major peak at a retention time of 12.1 min (Fig 44). The HPLC profile of compound 3 contains several smaller peaks which is characteristic of natural constituents in aloe chromatograms (Zonta et al., 1995).

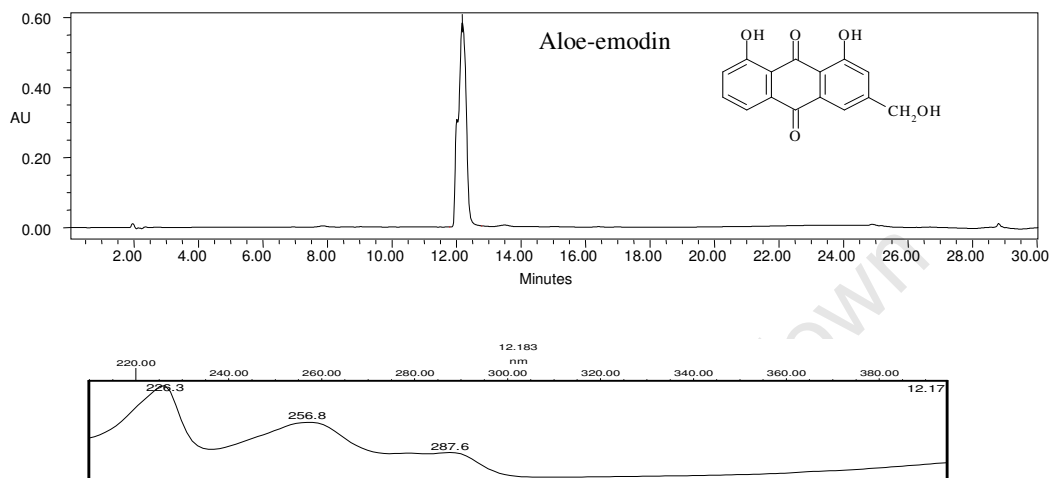


Figure 45 HPLC profile of AE on a C18 RP column ( $\lambda = 250$  nm)

HPLC conditions: Mobile phase  $\text{H}_2\text{O}:\text{ACN}$  using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

Aloe-emodin (Sigma) is commercially available and was used for bioactivity testing and comparative purposes during the analytical investigation of compound 3. Compound 3 showed similarities in the UV spectra and retention time to the known compound aloe-emodin (AE) (Fig 45). LC-MS analysis showed that the molecular ion of AE ( $m/z$  269) is present in compound 3 (Figures 46 and 47). AE is a degradation product of aloin thus its presence in *A. ferox* is expected (Saccù et al., 2001). The MS data identified compound 3 as AE.



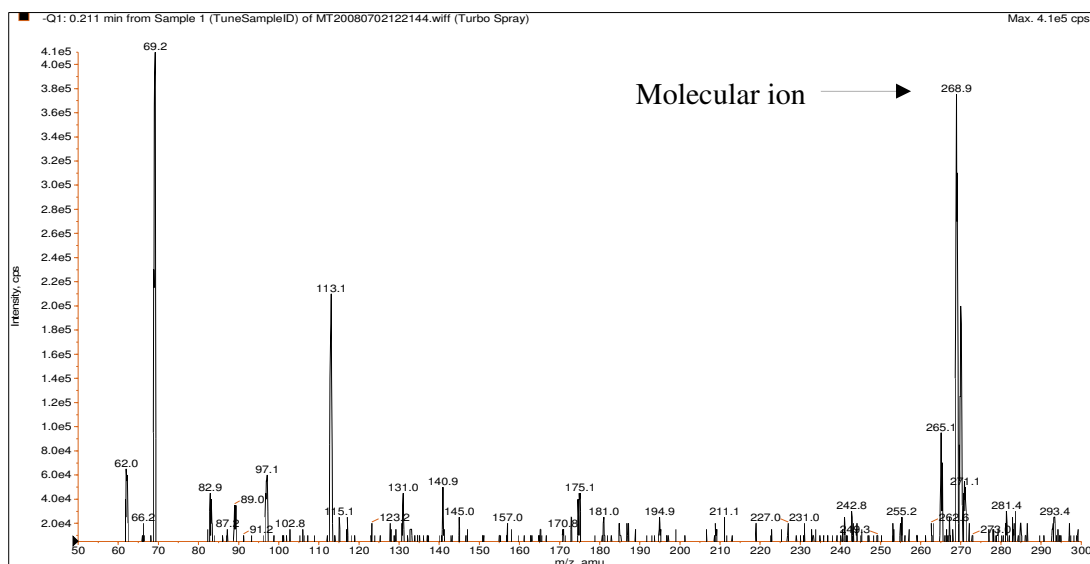


Figure 46 LC-MS spectrum of AE

LC-MS conditions: Negative ionization in MP ACN:0.1% formic acid (1:1), infused 5  $\mu$ l.

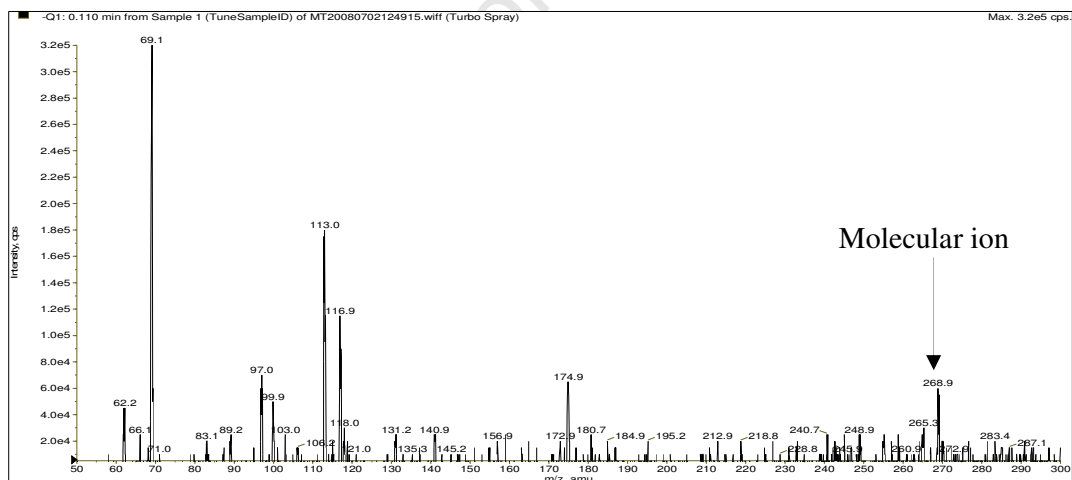


Figure 47 LC-MS spectrum of compound 3

LC-MS conditions: Negative ionization in MP ACN:0.1% formic acid (1:1), infused 10  $\mu$ l.

Compound 4 was obtained by combining CC-fractions 51 – 55. HPLC was used to determine the purity of compound 4. The UV spectrum of this compound was compared to published data. The spectral data of compound 4 was identical to two known compounds namely aloesin and aloeresin (Zonta et al., 1995). The absorbance of aloesin at 250 nm is stronger than the absorbance at 300 nm; while the opposite applies to aloeresin. The UV-spectrum obtained for compound 4 revealed that the absorbance at the 251 nm band is higher than the 289.9 nm band, which compares well with aloesin (Fig 48).

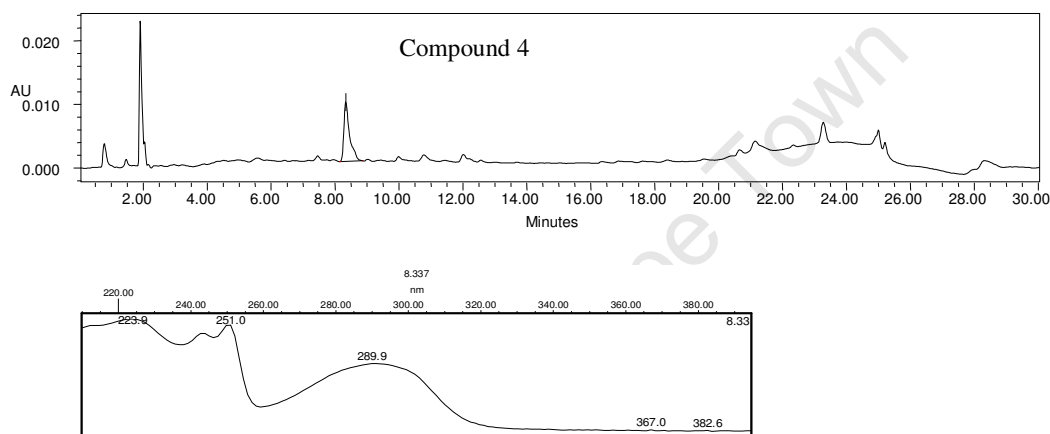


Figure 48 HPLC profile of compound 4 on a C18 RP column ( $\lambda = 280$  nm)

HPLC conditions: Mobile phase H<sub>2</sub>O:ACN using a gradient of 10 – 100% ACN (20 min), 100% ACN hold (2 min), 100 – 10% ACN (3 min), 10% ACN hold (5 min).

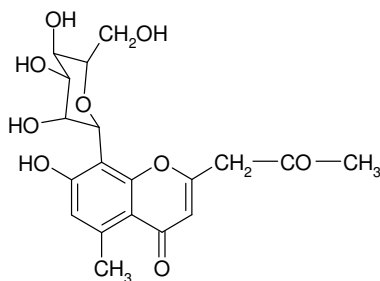


Figure 49 Chemical structure of Aloesin

Aloesin is a C-glycosylchromone with a molecular formula of  $C_{19}H_{22}O_9$  (Fig 49) (Haynes et al., 1970). Aloesin has a molecular ion of  $m/z$  394 which was used for identification via LC-MS analysis. The molecular ion ( $m/z$  394) was present in compound 4 but relatively high background signals showed that the compound contains impurities (Fig 50). The LC/MS data did provide further evidence that the compound is not aloeresin. No standard was available for comparative testing. LC-MS/MS analysis of  $m/z$  394 revealed product ions  $m/z$  324.8, 230 – 240,  $\pm 200$ ,  $\pm 170$ , 62.2 and 34.8 (Fig 51). Previous studies showed similar product ions for aloesin (Dell'Agli et al., 2007). The fragmentation pattern confirmed that compound 4 is aloesin.

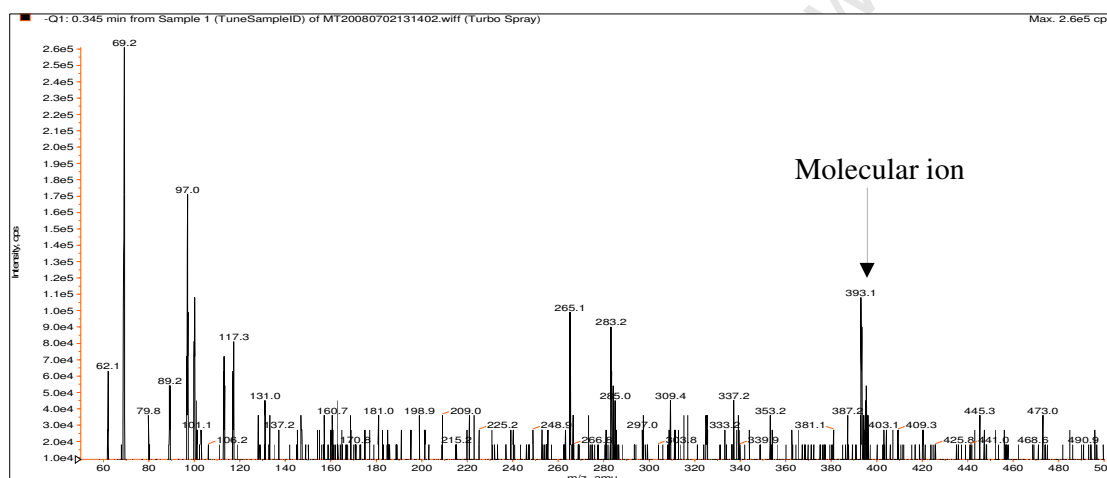


Figure 50 LC-MS spectrum of compound 4.

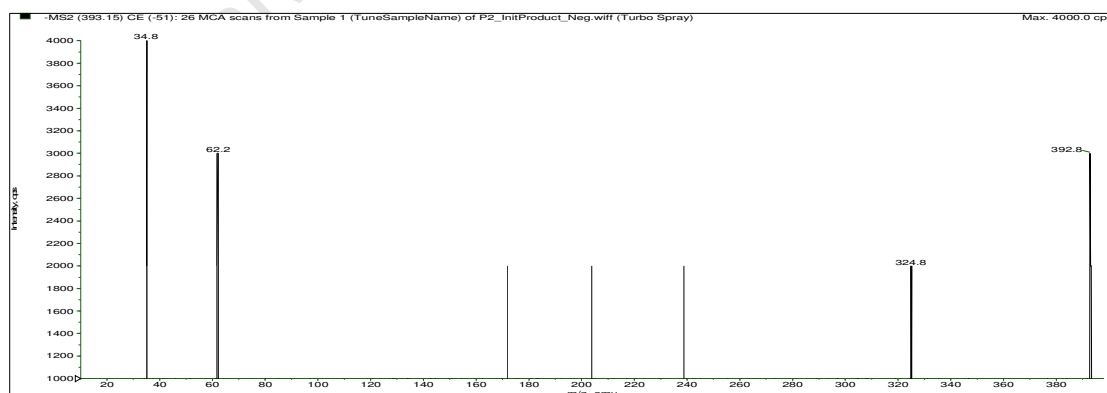


Figure 51 LC-MS/MS spectrum of  $m/z$  394

LC-MS conditions: Negative ionization in MP ACN:0.1% formic acid (5:5), infused 10  $\mu$ l.

### 5.3 *In vitro* bioactivity of compounds 1 – 4

The test compounds were screened for *in vitro* antiplasmodial activity against the CQS D10 strain of *P. falciparum* as described in Chapter 7 (7.6). Cytotoxicity screening was done using the CHO cell-line and antimycobacterial testing was done against *M. aurum* A<sup>+</sup> according to methods in 7.7 and 7.9 respectively. IC<sub>50</sub>-values are given as a mean value with standard deviations of 2 independent experiments.

Table 27 IC<sub>50</sub>-values of compounds against the CQS D10 strain of *P. falciparum* and cytotoxicity of compounds (±SD)

Sample	Antiplasmodial	Cytotoxicity	SI <sup>a</sup>
	D10: IC <sub>50</sub> (µg/ml)	CHO: IC <sub>50</sub> (µg/ml)	
Compound 1	2.92 ±0.01	50.58 ±5.94	17.32
Compound 2	49.09 <sup>b</sup>	–	–
Compound 3	> 100 <sup>b</sup>	–	–
Compound 4	12.51 <sup>b</sup>	> 100	ND
Aloe-emodin	> 100	–	–
Chloroquine	16.26 ±2.42	–	–
Emetine	–	0.06 ±0.0	–

Selectivity index (SI<sup>a</sup>) = cytotoxicity IC<sub>50</sub>/antiplasmodial D10 IC<sub>50</sub>

b = tested on one occasion in duplicate

ND = not determined

Compound 1 showed moderate activity with an IC<sub>50</sub>-value of 2.9 µg/ml (Table 27). Cytotoxicity results revealed a SI of 17 which indicates that the potency of the compound is selective for the malaria parasite. Compound 2 did not show activity (IC<sub>50</sub> = 49.09 µg/ml). Compound 3 and AE were not active against the CQS D10 strain of *P. falciparum* with IC<sub>50</sub>-values higher than 100 µg/ml. Compound 4 was slightly active against the CQS strain of the parasite with an IC<sub>50</sub>-value of 12.5 µg/ml. AE has been reported to have moderate antibacterial activity against *Micrococcus kristinae*, *B. cereus*, *B. subtilis*, *S. aureus*, *S. epidermis*, *E. coli* and *Shigella sonnei* (Kambizi et al., 2004 and Cooposamy et al., 2006). This is the first time to our knowledge that AE was tested for *in vitro* antiplasmodial and antimycobacterial activity.

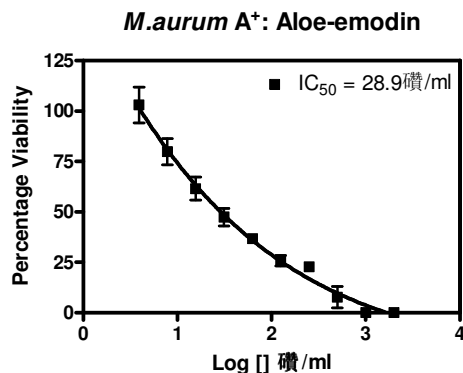


Figure 52 Dose-response curve of Aloe-emodin against *M. aurum* A<sup>+</sup>

A MIC of 31.25 µg/ml was determined based on visual growth of *M. aurum* A<sup>+</sup>. The compound was further tested to establish an IC<sub>50</sub>-value. AE showed moderate activity against *M. aurum* A<sup>+</sup> with an IC<sub>50</sub>-value of 28.9 µg/ml (Fig 52).

#### 5.4 *In vivo* antiplasmodial activity

The DCM extract of the whole leaf was investigated for *in vivo* antiplasmodial activity according to 7.10 of Chapter 7. The CQS *P. berghei* ANKA and CQR *P. yoelii* NS strains were used in a four-day suppressive test. Plant extract was prepared to 400 mg/kg bw in 8% ethanol diluted with 5% sodium bicarbonate and orally administered. Three groups of four mice each were tested against the CQS strain and three groups consisting of three mice were tested against the CQR strain. Averaged parasitemia were compared to the untreated control group to assess activity. The crude extract did not show suppression of parasitemia on day 4 against the CQS *P. berghei* strain (Fig 53). A suppression of parasitemia was observed against the CQR *P. yoelii* strain.

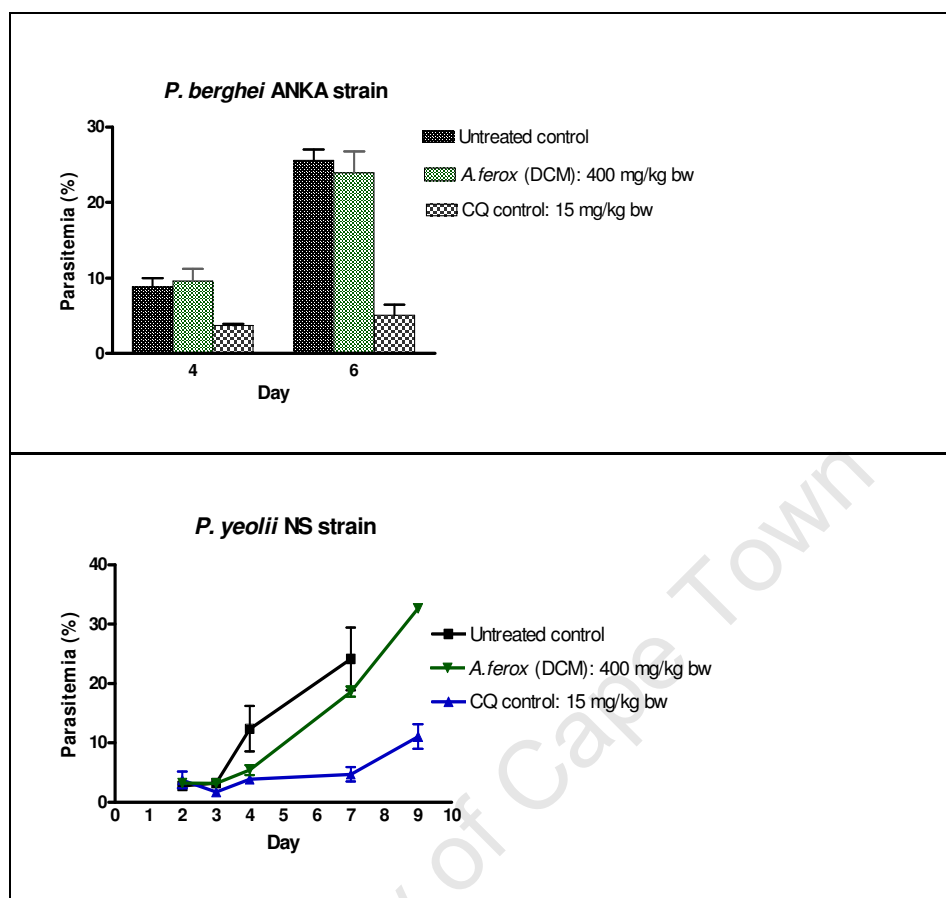


Figure 53 *A. ferox*: Average percentage parasitemia using 4-day suppressive test against the CQS *P. berghei* ANKA strain and CQR *P. yeolii* NS strain

Table 28 Percentage growth inhibition of *A. ferox* against the *P. yeolii* NS strain

Days post-infection	<i>A. ferox</i> (DCM)	CQ control
4	55.91 %	68.82 %
7	22.90 %	80.41 %

Inhibition of parasitemia of 55.9% was observed after 4 days of treatment against the CQR *P. yeolii* NS (Table 28). The parasitemia against the CQR strain increased steadily after termination of treatment on day 4. No toxic effects were observed for the crude extract.

## 5.5 Discussion

### 5.5.1 *In vitro* bioactivity

The winter collections of *A. ferox* were four times more active against the CQS strain of *P. falciparum* than the summer collections. Temperature and rainfall play an important role in the development and metabolism of plants (Trease and Evans et al., 1996). A higher percentage aloin, the major compound in the dried leaf juice, was observed during summer than winter months (Trease and Evans et al., 1978). Previous studies showed that aloin is inactive against the malaria parasite (van Zyl et al., 2002). The decrease in aloin-type constituents during winter months might account for the increased antiplasmodial activity. The inner gel contains several compounds which are believed to be biologically active (van Wyk et al., 1996 and Loots et al., 2007). Therefore the leaves collected in winter were separated into the gel and outer leaf. The whole leaf extractions were more active against *P. falciparum* than the gel and outer leaf separated. Various studies have focused on the bioactivity of the inner gel of *Aloe* species as opposed to the whole leaf.

An enhancement in activity was observed with fractionation. Bioassay-guided fractionation of the whole leaf extract yielded two compounds. Compound 1 was the most active against the CQS strain of *P. falciparum* with an  $IC_{50}$ -value of 2.9  $\mu\text{g/ml}$ . The chemical structure of compound 1 was not determined due to insufficient material. LC-MS analysis suggested a mass ion of  $m/z$  404 for compound 1. Compound 2 was inactive with an  $IC_{50}$ -value of 49.1  $\mu\text{g/ml}$ . The HPLC evaluation of compound 2 showed that it degraded in solution thus no structure determination was done. The absorbance data suggests compound 2 has a chromone-like structure (Zonta et al., 1995). TLC separation of the gel and outer leaf extracts enhanced antiplasmodial activity. The activity of the outer leaf extracts ranged from an  $IC_{50} = 80 - 100 \mu\text{g/ml}$  but the fractions showed increased activity ranging from  $IC_{50} = 2 - 9 \mu\text{g/ml}$ . This trend in activity led to further investigation of the outer leaf extract which yielded two major compounds (3 and 4). The HPLC profiles of compound 3 and AE were identical. The identity of compound 3 as AE was further confirmed via LC-MS analyses. AE was present in compound 3. Compound 3 and AE did not show

antiplasmodial activity. AE was moderately active against *M. aurum*. HPLC and LC-MS analyses assigned compound 4 as the known compound aloesin. Compound 4 was slightly active against the CQS strain of the parasite and showed no cytotoxicity. Metabolite studies of aloesin showed that the C-glycosyl bond is cleaved by human intestinal bacteria (Che et al., 1991). Aloesin (m/z 394) is converted to two metabolites aloesone (m/z 232) and aloesol (m/z 234). LC-MS/MS analysis of compound 3 (m/z 394) showed product ions similar to published data (Dell'Agli et al., 2007). Degradation of aloe constituents is a common phenomenon during phytochemical investigations (Zonta et al., 1995). It is possible that compounds 1 and 2 might be degradation products or derivatives of known compounds.

### 5.5.2 *In vivo* activity

*In vivo* activity is influenced by pharmacodynamic and pharmacokinetic properties which might enhance or reduce activity (Kirby et al., 1996). *In vitro* screening in combination with an *in vivo* evaluation is essential in determining the bioactivity profile of a plant extract. Several examples exist of compounds and plants which exhibit *in vitro* activity but lack *in vivo* activity and vice versa (Phillipson et al., 1993). Therefore the crude extract of *A. ferox* was investigated for *in vivo* antiplasmodial activity. The crude extract was more active *in vivo* against the CQR strain than the CQS strain of the parasite. The crude extract inhibited parasitemia by more than 50% on day 4 of treatment against the CQR strain of *Plasmodium*. Interestingly the crude extract was less active *in vitro* against the CQR strain than the CQS strain of *P. falciparum*. The crude extract did not show *in vivo* toxicity at the dose administrated.



# *Chapter 6*

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## *Conclusion*

University of Cape Town

## 6. Conclusion

### 6.1 *Siphonochilus aethiopicus*

#### 6.1.1 *In vitro* bioactivity

The traditional use of *S. aethiopicus* (wild ginger) for the treatment of malaria and malaria-like symptoms initiated this scientific investigation. The *in vitro* antiplasmodial investigation of the EA extract revealed activity against *P. falciparum*. Bioassay-guided fractionation resulted in the isolation of three structurally-related furanoterpenoids. Chemical analogues of natural products are often obtained from the original plant as many compounds exist as groups of structurally-related metabolites within a species or related species and genera of one or more families (Kirby et al., 1996). These naturally occurring metabolites can provide structure activity relationships which might explain the source of activity. This class of compounds has been isolated from the Zingiberaceae family (Hikino et al., 1968). A furanosesquiterpenoid, furanodiene, was isolated from *Curcuma zedoaria* (Zingiberaceae). Similar furanosesquiterpenoids have been previously isolated from marine sources (Yamakawa et al., 1975; Mollo et al., 2005 and Iguchi et al., 1986). In a recent publication by Pink et al., (2005) the criteria for selecting a hit compound was specified as follows:

- *In vitro* activity with an  $IC_{50}$ -value  $\leq 1 \mu\text{g/ml}$
- Selectivity index  $\geq 10$  fold

According to these criteria the compounds isolated did not show significant *in vitro* antiplasmodial activity for them to be considered as hit compounds. However the selectivity of the compounds towards the CQR K1 strain compared to the CQS D10 strain of *P. falciparum* is noteworthy. The observed selectivity towards the CQR strain indicates that these compounds act by a mechanism unrelated to CQ. The reason for this activity is not known. The activity of the EA extract of *S. aethiopicus* and compound 3 is comparable against both strains of *P. falciparum*. Compound 3 and the EA extract showed a two-fold increase in activity towards the CQR K1 strain with similar SI's. The RI of compound 2 showed an increase in activity towards the

CQR K1 strain compared to compound 1. Interestingly, the only difference in the structure of compound 1 and 2 is the substitution of the OH-group with hydrogen which results in a three-fold increase in activity against the CQR K1 strain. Introduction of an additional double bond in ring C (compound 3) further improves activity against both strains. It is possible that the antiplasmodial activity of these compounds can be attributed to the furan moiety in the structure. Recently, two privileged substructures, a 2(5H)-furanone and a dihydrofuran-4-one moiety were identified as the possible chemical entities responsible for antiplasmodial activity of hirsutinolides isolated from *Vernonia staehelinoides* (Pillay et al., 2007). The mechanism of action of this class of antiplasmodial compounds is not known. The *in vitro* antiplasmodial data suggests that the activity of the crude plant extract is due to compound 3. Compounds 2 and 3 are more active against the chloroquine resistant strain of *P. falciparum* than the chloroquine sensitive strain. The structure-related increase in antiplasmodial activity against the CQR strain suggests that the basic structure of these compounds may be useful as a scaffold for further synthesis of derivatives. However the SPE fractions were more active than the compounds isolated, indicating that the activity of the crude extract might be due to the synergistic effect of different components. This study provides evidence of antiplasmodial compounds present in this plant which may play a role in the traditional use of *S. aethiopicus* in the treatment of malaria.

Previous studies on a crude extract of this plant showed antibacterial activity against *S. aureus* and *K. pneumoniae* (Stafford et al., 2005). Broad spectrum activity against a range of microorganisms is essential to fully evaluate the potential of novel compounds and traditional remedies. The semi-pure fraction consisting of all three compounds was extracted using a pH 11 buffer. This fraction was tested against *M. aurum* which serves as a preliminary antimycobacterial test for *M. tuberculosis*. The fraction was found to be moderately active. No activity was found with the isolated compounds against *M. tuberculosis*, *S. aureus*, *K. pneumoniae* and *C. albicans*. The process of bioassay-guided fractionation might reveal more active compounds as it was not used as an approach for the antimycobacterial, antibacterial and antifungal testing.

### 6.1.2 *In vivo* schizontocidal activity

*In vivo* activity is influenced by pharmacokinetic and pharmacodynamic properties of the drug. The promising *in vitro* activity of the EA extract and compound 3 are selective to the malaria parasite particularly the CQR strain of *P. falciparum*. The SPE fractions showed greater *in vitro* activity than the three isolated compounds which suggested that the antiplasmodial activity might be due to synergism rather than a single compound. All *in vivo* experiments were performed against the CQR *P. yeolli* NS strain due to the observed *in vitro* selectivity towards the CQR strain. The *in vivo* schizontocidal activity of the EA extract and compound 3 were evaluated in a 4-D suppressive test. HPLC analysis showed that compound 3 is one of three major compounds in the EA extract. The EA extract suppressed parasitemia during treatment but not after the termination of treatment. Compound 3 also showed a suppression of parasitemia on day 4. The EA extract was administered 4-fold higher dose than compound 3. However the EA extract inhibited parasite growth more than compound 3 at the administered dosages. These results supported the hypothesis that more than one compound can be responsible for the antiplasmodial activity of the *S. aethiopicus*. An *in vivo* study to compare the schizontocidal activity of the EA extract and isolated compounds at the same dose will be helpful to draw further conclusions.

The schizontocidal activity of the EA extract during the 4-D treatment was further investigated using a 7-D suppressive test. Drug formulations can have an effect on bioavailability and pharmacokinetics resulting in either increased or decreased *in vivo* schizontocidal activity (Peters et al., 2002). In the 7-D test the activity of the EA extract in two formulations, an aqueous-based 5% SB (with addition of 5% ethanol) and lipid-based SMEDDS formulation, were compared at the same dose. Suppression of parasitemia on days 4 and 7 was observed with the 5% SB formulation. The SMEDDS formulation did not show any suppression of parasitemia in the 7-D test. Interestingly the parasitemia remained unchanged from day 5 to 7 with the SMEDDS formulation. No parasite clearance was observed with the EA extract in a curative experiment. In a prophylactic study of *S. aethiopicus* (EA) a weak to moderate effect was observed. The dose formulation of the EA extract had an effect on its *in vivo* activity. The EA extract in the 5% SB formulation suppressed parasitemia when

treated for four or seven days. This study gives support to the traditional use of wild ginger for the treatment of malaria.

Lipophilic quassinoids isolated from *B. javanica* have promising antiplasmodial activity but were also very toxic (O'Neil et al., 1987). This medicinal plant like *A. annua* has been used as a traditional medicine for centuries in an aqueous decoction. Aqueous decoctions can contain lower amounts of active constituents and the reduced concentration can lead to less toxicity (Kirby et al., 1996). The route of administration and traditional preparation of traditional medicine plays an important role in tolerability and efficacy. Wild ginger is traditionally used as an infusion or the fresh rhizomes and roots are chewed. This part of the study focussed on isolating novel antiplasmodial compounds thus a selective approach was used in evaluating the plant material. Scientific investigations using the traditional formulations might reveal better activities as more compounds might be available.

### **6.1.3 *In vivo* bioavailability and metabolism**

LC-MS/MS technology has revolutionized bioanalytical analyses and has become the method of choice for evaluation of pharmacokinetic studies. Advances in bioanalytical methods over the past decade have paved the way forward for techniques that are higher in throughput and sensitivity (Shah et al., 2000). The criteria for method validation recommended by the Food and Drug Association Guidance for accuracy, precision, selectivity, sensitivity, reproducibility and stability serves as a guideline for bioanalytical method development of small molecules (Bansal et al., 2007). These parameters were taken into account during method development. A novel method to evaluate *in vivo* pharmacokinetic factors such as absorption and metabolism was developed using a mouse-model. The use of small animals for pharmacokinetic studies is ideal for plant-derived compounds as these studies are often neglected due to insufficient quantities. LC-MS/MS analyses were performed using only 10 µl of plasma. The small sample size needed for analysis eliminated the sacrifice of animals at time points. Thus a small number of animals were used.

The absorption and metabolism of a compound can influence its *in vivo* activity and toxicity. If a drug is poorly absorbed it may not be available or may be available in low concentrations at the target site. Hence a compound which is active *in vitro* but not active *in vivo* such as compound 3 might be as a result of poor absorption. Metabolism within an *in vivo* system involves a variety of enzymatic pathways and the production of numerous novel compounds at varying concentrations. These newly formed metabolites can increase or decrease the activity of a compound. The pharmacokinetic study was designed two-fold, the first study investigated the bioavailability of compound 3 and the second study investigated the metabolism of compound 3.

The *in vivo* bioavailability of compound 3 was investigated using an aqueous- and lipid-based SMEDDS formulation. The formulations were compared using oral and SC routes of administration. The bioavailability study of compound 3 revealed that the SMEDDS formulation administered either orally or subcutaneously, is better absorbed than the DMSO formulation. The SC route of administration resulted in higher plasma levels. A delayed trend in absorption was observed with the SC administration of compound 3 in the SMEDDS formulation.

The bioavailability of the EA extract was investigated to determine if administering the mixture would result in better absorption of compound 3. The EA was orally administered at a low dose of 400 mg/kg bw and a high dose of 800 mg/kg bw in SMEDDS formulation. Compound 1 is the most abundant constituent in the EA extract and was absorbed in greater quantities than compounds 2 and 3. The SMEDDS formulation facilitates a delayed effect on absorption of compound 3 given either alone or in the EA extract. The slower release of compounds was not evaluated as no blood samples were taken beyond 5 hrs. The disadvantage of a small animal study design is limitation of blood sampling intervals. The plasma levels of compound 3 in the EA extract was higher than that of the pure compound. The EA extract was more active *in vivo* than compound 3 against the CQR *P. yeolli* strain. Improved absorption of compound 3 was observed with the EA extract which might explain the increased *in vivo* activity of the extract. The bioavailability data suggests that compound 3 has poor absorption especially if given alone. Consequently the reached

plasma levels of compound 3 are three times less than the concentration needed to inhibit 50% of parasite growth and this could account for its weak *in vivo* activity.

The lack of *in vivo* activity of compound 3 can also be explained by rapid metabolism and the subsequent formation of inactive metabolites. Metabolism plays an important role in drug availability and thus efficacy. A preliminary metabolism study was performed by oral administration to assess the effect of phase I metabolism on compound 3. CYP is a superfamily of enzymes responsible for phase I hepatic metabolism and the most important subfamilies in the human genome are CYP 1A, CYP 2C, CYP 2D and CYP 3A (Granvil et al., 2003, Willcox et al., 2004 and Dresser et al., 2000). Recent studies reported that the CYP enzyme system plays an important role in the biosynthesis of  $\beta$ -substituted furan moiety in furanoterpenes (Gaikwad et al., 2002). Therefore it is likely that this class of compounds can undergo enzymatic metabolism by CYP enzymes. Compound 3 was not detected in the matrixes (blood, urine and faeces) evaluated for the metabolism study. A more intense investigation might reveal metabolites as this metabolite study had time and sample constraints. The precursor experiment was conducted to evaluate the presence of the major product ion. The expected fragmentation was not observed with this experiment. Poor oral bioavailability is generally associated with substrates that have a high phase I elimination. The metabolite study further provided evidence that compound 3 probably undergo phase I metabolism. The compound might be metabolized by novel pathways thus the predicted metabolites could not be detected. No neutral loss experiments to search for expected metabolites were performed due to the lack of visible metabolites during the precursor experiment. Further fine-tuning of the LC-MS method and more advanced technologies such as specialised software packages in metabolite identification may reveal better results. This study could not detect visible metabolites of compound 3 but provides a platform for future work.

Different compounds in a crude extract can act as an inhibitor of metabolic enzymes resulting in higher plasma concentrations. This phenomenon can shed light on the increased absorption of compound 3 from the EA extract. The pharmacokinetic investigation suggested that the weak *in vivo* activity of compound 3 is due to poor absorption rather than rapid metabolism. Pharmacokinetic studies performed early in

drug development will aid in making enlightened decisions about promising candidates.

## 6.2 *Aloe ferox*

Environmental conditions play an important role in the development and metabolism of plants (Trease and Evans et al., 1996). The effect of continuous rain can lead to leaching of water-soluble components from the leaves and roots. This phenomenon has been observed with alkaloid-, glycoside- and volatile oil-producing plants and could explain a decrease of active constituents in winter. However the opposite was observed in this study. The most potent extraction solvent was DCM which extracts non-polar constituents while the water extract showed no antiplasmodial activity. This indicates that the active components of the plant are non-polar. Lesser activity in summer collections can be accounted for by the effect of dry and stressed summer conditions which might yield fewer amounts of the active constituents. Studies on *A. vera* have shown that the barbaloin content and distribution in leaf parts may play a role in the plant's defence mechanism against herbivores (Esteban-Carrasco et al., 2001). In this study higher amounts of aloin-type compounds were observed in the summer collections. The results support the general rule supporting the collection leaves during the flowering season. Chemical similarities were observed with plant extracts from two different locations which corresponded with previous studies by Viljoen (2001).

Two unknown compounds were isolated from whole leaf DCM extracts. Compound 1 was the most active compound against the CQS strain of *P. falciparum* and showed little cytotoxicity. The molecular ion of compound 1 ( $m/z$  404) was determined. The structure elucidation of compound 1 was not determined due to insufficient material. Compound 2 was inactive against the CQS strain of *P. falciparum*. HPLC data showed that compound 2 has a chromone-like structure. Two known compounds, aloe-emodin and aloesin, were isolated from the outer leaf extract. Aloin is a known pro-drug for AE thus the presence of aloin in the DCM extract can account for the consequent isolation of AE. The identity of compound 3 as AE was confirmed via



HPLC and LC-MS analyses. AE did not show antiplasmodial activity and was moderately active against *M. aurum*. Aloesin was slightly active against the CQS strain of the parasite and showed no cytotoxicity. Compounds 1 and 2 might be degradation products or derivatives of known compounds as degradation products often occur in aloes (Zonta et al., 1995). Fractionation of the DCM extracts showed an enhancement in activity *in vitro* but only one of the four compounds isolated was active. There might be other compounds present in the crude extract which contributes to the antiplasmodial activity or the activity might be caused by a collective approach of different components instead of one compound. Aloe gel components have been shown to work synergistically to address inflammation and wound healing (Lindsey et al., 2002).

*In vivo* screens are an imperative part of bioactivity testing as it might lead to the enhancement or reduction in activity (Kirby et al., 1996). Studies showed that berberis alkaloids have weak *in vitro* activity against *E. histolytica* but have significant *in vivo* activity (Subbaiah et al., 1967 and Kirby et al., 1996). The whole leaf DCM extract was further investigated for *in vivo* schizontocidal activity. The DCM extract suppressed parasitemia on day 4 of treatment against the CQR strain but had no effect on day 4 against the CQS strain of *Plasmodium*. Thus the DCM extract revealed greater suppression of parasitemia against the CQR strain than the CQS strain of the parasite. These results did not correspond with the *in vitro* activity of the crude extract which was more active against CQS strain of the parasite. Studies have shown that *in vitro* and *in vivo* activity does not always correlate (Phillipson et al., 1993). No toxicity was observed for the crude extracts with *in vitro* or *in vivo* testing. It is evident by the wide range of traditional use of Aloes that its effect may not be selective to an organism but address a wide range of symptoms.

### 6.3 Recommendations

The furanoterpenoid skeleton of the compounds isolated from *S. aethiopicus* can be used as a scaffold for further chemical modifications. The synthesis of tricyclic furanoterpenoids and the possible biosynthetic route for the formation of the furan skeleton in lower furanoterpenoids has been investigated (Moiseenkov et al., 1997 and Gaikwad et al., 2002). The synthesis of the furanoterpenoid skeleton and derivatives might be possible through a synthetic and biosynthetic route. The synthesis of derivatives will be to produce antiplasmodial compounds with enhanced *in vitro* and *in vivo* activity and low cytotoxicity. The bioavailability of active derivatives can be tested *in vivo* using the methods developed in this study. The separation methods developed for *S. aethiopicus* will be useful to further isolate compounds from this plant. Additional *in vitro* experiments to evaluate the effect of the isolated compounds as well as the EA extract in combination with CQ on CQR strains of *P. falciparum* can be conducted. *In vivo* schizontocidal activity of the crude extract and antiplasmodial compounds can be further investigated. The bioactivity testing of novel compounds against a wide variety of organisms should be encouraged to effectively evaluate potential hit compounds. A comparative study to evaluate oral and intravenous administration might help to understand if it undergoes phase I or phase II metabolism.

### 6.4 Research outputs

- Oral presentation of part of thesis: South African Pharmacology & Toxicology Congress, 2007.
- Oral and poster presentation of part of thesis at the Young Scientist Workshop: 7<sup>th</sup> Joint Meeting of AFERP, ASP, GA, PSE and SIF, 2008.
- The bioactivity activity of novel furanoterpenoids isolated from *Siphonochilus aethiopicus*. Authors: Carmen Lategan, William Campbell, Tracy Seaman and Peter J. Smith. *Article in press*: Journal of Ethnopharmacology.

# *Chapter 7*

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## *Materials and Methods*

## 7. Materials and Methods

### 7.1 Plant collection

*A. ferox* was collected at Kirstenbosch botanical gardens and Buffelsfontein farm, Albertinia in the Western Cape (South Africa). Collection was done in summer and winter. Chris Cupido, a botanist at the Kirstenbosch Research Centre (Cape Town) verified the collected plant material. A voucher specimen (Lategan 1) was submitted to the Compton herbarium at Kirstenbosch national botanical gardens (Cape Town). *S. aethiopicus* was initially donated by Nigel Gericke (South African National Biodiversity Institute, Durban). Plant material was subsequently supplied by the New Guelderland sugar estates in Kwazulu-Natal, South Africa (batch number 200/ngse 02) and was collected in October 2002. The rhizomes were received in a freeze dried form and ground to a fine powder. A voucher specimen (barcode number 3667) was submitted to the Bolus herbarium at the University of Cape Town. Plant material was stored at room temperature until extraction.

### 7.2 Extraction of plant material

The preparations of extracts were not done according to the traditional use. The drying process of plants results in the rupture and degradation of cell membranes which can lead to the extraction of more compounds (Stafford et al., 2005). Plant material was extracted using various solvents, filtered and the filtrate reduced under pressure using a Büchi rotavapor R-205 (Büchi Labortechnik AG Schweiz). Extraction solvents were selected based on previous studies (Clarkson et al., 2004 and Toyer et al., 2005). *A. ferox* leaves, leaf exudate and cold H<sub>2</sub>O extractions were freeze-dried using a DURA-DRV II instrument (FTS SYSTEMS, NY USA). The dried extracts were stored at -20°C.

## 7.3 Fractionation and purification of compounds from *S. aethiopicus*

### 7.3.1 Solid Phase Extraction

SPE was done on a 70 ml reverse phase C18 Isolute column cartridge (2.6 x 13.0 cm, 10 g sorbent, IST Ltd, Anatech, South Africa). The extract was dissolved in ACN and water and loaded onto the column. A previous study showed that the 60% ACN and 80% ACN fractions were the most active (Toyer et al., 2005). The extract was eluted with a gradient of decreasing polarity (ACN:H<sub>2</sub>O) with 10% increments of ACN starting from 50:50 (ACN:H<sub>2</sub>O) to 100:0 (ACN:H<sub>2</sub>O). A vacuum was applied to the cartridge and the fractions collected were concentrated under reduced pressure followed by freeze drying to displace water. This procedure was repeated several times to obtain sufficient material for further fractionation.

### 7.3.2 Liquid-liquid extraction

This procedure was done to scale-up the fractionation process of the crude extract of *S. aethiopicus*. Britton Robinson universal buffers (pH 2 – 12) were prepared with solvent A (6.005 g/L acetic acid, 11.53 g/L phosphoric acid and 6.183 g/L boric acid) and solvent B (0.5 M NaOH).

Method development: A 10 mg/ml stock solution of the crude extract was prepared in EA. 100 µl of the stock solution was added to 1 ml of the respective pH buffer and 5 ml EA. This mixture was vortexed, centrifuged at 13 000 rcf for 5 min and the supernatant removed. The organic solvent was removed using a cold trap system (Jouan RC 10.10). The dried material was reconstituted in methanol or DMSO and evaluated using HPLC.

Optimisation of extraction: A stock solution of ±60 mg/ml of the crude extract in EA was prepared. Then 10 ml of the stock solution was added to 50 ml pH 11 buffer and 250 ml EA (5 x volume of pH buffer). The mixture was shaken for 15 min and the two layers were separated and dried using a rotary evaporator and freeze dryer.

### 7.3.3 High Performance Liquid Chromatography

HPLC was done using a Waters 1525 system consisting of a binary HPLC pump, a photodiode array detector, a fraction collector and an auto sampler controlled by Millennium v.4.0 software. Alternatively, a Shimadzu LC10AS high pressure system consisting of binary LC10AS pumps, a diode array detector and an auto sampler controlled by Shimadzu software was used. All solvents were of analytical grade (Sigma-Aldrich, South Africa) and Millipore water (Milli-Q water system) was used.

#### 7.3.3.1 Analytical HPLC

The UV absorption was detected with a photodiode array detector. Analytical HPLC profiles were obtained using a Supelco Discovery RP C18 (15 cm x 4.6 mm, 5  $\mu$ m) column with a C18 guard column (5  $\mu$ m). The flow rate was set at 1 ml/min and 50  $\mu$ l of a 1 mg/ml sample concentration in methanol, ACN or DMSO was injected onto the column. The mobile phase H<sub>2</sub>O:ACN was run at a gradient of 10% ACN to 100% ACN in 20 min with a hold at 100% ACN for 2 min, then reduced to 10% ACN in 3 min and kept at 20% for 5 min. The gradient was set according to this method unless stated otherwise.

#### 7.3.3.2 Semi-preparative HPLC

HPLC collections were performed on a semi-preparative Supelco Discovery C18 (25 cm x 10 mm, 5  $\mu$ m) column attached to a C18 guard column (5  $\mu$ m) with the flow rate adjusted to 2 ml/min. The diode array detector was set to UV absorption at  $\lambda = 254$  nm as all three peaks were clearly detected using this wavelength. The mobile phase was adjusted to a gradient of 40% ACN to 100% ACN in 20 min with a 2 min hold at 100% ACN, then reduced to 40% ACN in 3 min and conditioned at 40% for 5 min before the next run. Samples of 10 mg/ml were prepared and 50  $\mu$ l was injected onto the column during method development. Further optimisation for large scale collection resulted in the injection of 50  $\mu$ l at a 100 mg/ml sample concentration.

### 7.3.4 Analytical methods for structure elucidation

The NMR spectra were performed on a Varian Unity Inova 600 MHz and accurate mass determination was recorded on a Waters API Q-TOF Ultima system (University of Stellenbosch, Stellenbosch). Optical rotations were done on a Perkin-Elmer 241 polarimeter at 589 nm (Na D-line) using a 1,001 dm cell (CSIR, Pretoria). The compounds were dissolved in methanol at a concentration of 10 mg/ml. The melting point was measured on a SANYO Gallenkamp variable electrical apparatus (United Scientific, Cape Town) and a Reichert-Jung Thermovar hot stage microscope (Department of Chemistry, University of Cape Town).

### 7.4 Fractionation and purification of compounds from *A. ferox*

SPE was performed as described above with an adjustment of 20% increments of ACN starting from 20:80 (ACN:H<sub>2</sub>O) to 100:0 (ACN:H<sub>2</sub>O). Additional separation using SPE was done with 5% increments of ACN starting from 40:60 (ACN:H<sub>2</sub>O) to 100:0 (ACN:H<sub>2</sub>O). Purity of fractions and compounds was evaluated via HPLC and TLC. HPLC conditions were the same as for *S. aethiopicus* analysis or adjusted to a gradient of 20% ACN to 100% ACN in 30 min with a hold at 100% ACN for 5 min, then reduced to 20% ACN in 5 min and kept at 20% for 2 min (7.3.3.1).

#### 7.4.1 Column chromatography

The outer leaf extract of *A. ferox* (DCM) was separated using column chromatography (silica gel type H, Sigma). Approximately 2g of the crude extract dissolved in DCM was loaded onto the column. The extract was washed with 100% petroleum ether (PE) and 15% EA in PE. Fractions were collected and pooled according to TLC analyses.

### 7.4.2 Chromatotron and thin layer chromatography

SPE fractions of *A. ferox* (DCM) whole leaf extract were further separated using a chromatotron (Harrison, Model 8924). The chromatotron is a preparative separation technique which is centrifugally accelerated. The sample was loaded onto a circular chromatotron plate which rotated under UV-light. Separation was performed on 1 mm plates (Silica gel PF<sub>254</sub> containing gypsum, Merck). Solvent systems of varying polarities were used to elute bands. The solvents form concentric bands which are collected together with solvent from the edge of the rotating plate. This method bypasses the time consuming step in TLC of scrapping-off bands, washing silica gel and filtering.

Chromatotron fractions of *A. ferox* were further fractionated using PTLC and analytical TLC techniques with toluene:ethyl acetate (93:7) as the MP. SPE fractions were also separated using TLC in MP developed by Duncan (2001).

### 7.4.3 Analytical methods for structure elucidation

Analytical methods using HPLC were used to determine the UV-absorption of compounds (7.3.3). An Applied Biosystems API-2000 mass spectrometer (MS) was used to determine the molecular ion of the compounds. Compounds were infused at 1 µg/ml in the both negative and positive ESI modes to select the molecular ion of interest. The best results were achieved in the negative ion mode infused in an acidic chemical environment (0.1% formic acid: ACN, 1:1, v/v) at a flow rate of 5 µl/min.

## 7.5 Preparation of samples for antiplasmodial and cytotoxicity assay

Chloroquine diphosphate (Sigma) was used as the reference drug for antiplasmodial assays. Emetine dihydrochloride (Sigma) was the reference drug for cytotoxicity assays. Stock solutions for test samples were prepared to 2 mg/ml. Test samples were dissolved in MeOH:H<sub>2</sub>O (1:9) or DMSO:H<sub>2</sub>O (1:9). A solvent control was carried out to ensure that the solvent concentration (0.5%) to which the parasites or cells were



exposed did not have an effect. The highest concentration of methanol (0.5%) to which the parasites or cells were exposed had no measurable effect on parasite survival

## **7.6 *In vitro* antiplasmodial testing**

### **7.6.1 Preparation of medium for culturing malaria parasites**

Parasites were maintained in medium containing 10.4 g/L RPMI 1640 with glutamine but without NaHCO<sub>3</sub> (BioWhittaker) supplemented with Albumax II (GibcoBRL) (5 g/L), 4 g/L glucose, 6 g/L Hepes (*N*-[2-hydroxyethyl]-piperazine-*N*1-[2-ethansulphonic acid]) (Sigma-Aldrich), 0.088 g/L Hypoxanthine, and 1.2 ml/L (0.05 g/L) gentamycin (Sigma-Aldrich). The RPMI contains a colour indicator, which appears orange at the desired pH. Culture medium or reagents were filtered under pressure firstly through a pre-filter (0.45 µm) to remove impurities followed by filter sterilization through a 0.22 µm filter. 5% sodium bicarbonate (8.4 ml) was added to 200 ml medium which was then referred to as complete medium. Wash medium was prepared and filtered the same as culture medium but did not contain albumax. The same amount of sodium bicarbonate solution was added to wash medium. D-sorbitol (5%) solution was filter sterilized. Medium and reagents were stored at 4°C.

### **7.6.2 Cultivation of malaria parasites**

The culture adapted CQS cloned D10 strain (derived from FCQ-27 from Papua New Guinea) (Ekong et al., 1993) was donated by Dr A.Cowman, Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia. The culture adapted CQR K1 strain (isolated at Kanchaburi, Thailand) (Thaithong et al., 1981) was donated by Dr D.Wallaker, University of Edinburgh, Edinburgh, Scotland.

Continuous *in vitro* cultures of asexual erythrocyte stages of *P. falciparum* were maintained using a modified method of Trager and Jensen (1976). All procedures were conducted under sterile conditions. O<sup>+</sup> human blood (Western Province Blood

Transfusion Service, Groote Schuur Hospital, Cape Town, South Africa) was washed twice with wash medium and centrifuged at 1200 rpm for 5 minutes. The supernatant was aspirated off and the pellet containing mostly RBC's was added to the parasites in the trophozoite stage. D-sorbitol was used to synchronise the parasites in the ring stage as the later stages incorporate more substances. The parasites were grown in tissue culture flasks (Greiner Bio-One) which were flushed with gas (3% O<sub>2</sub>, 4% CO<sub>2</sub>) for about 1 minute and incubated at 37°C.

After 24 hours the parasites were removed from the flask and centrifuged at 750 rcf for 3 minutes. The supernatant was removed and a thin blood smear was prepared from the pellet parasitized red blood cells (pRBC). The blood smear was fixed with methanol and stained with 10% Giemsa for microscopic evaluation. Complete medium (37°C) was added to pRBC, which was gassed and incubated at 37°C. Parasites were maintained at a parasitemia below 10%.

### **7.6.3 Cryostorage and thawing of malaria parasites**

Parasites were frozen in sterile freezing medium prepared with 1.6 g sodium lactate, 30 mg KCl, 1.38 g sodium dihydrogen phosphate, and 57 g glycerol in 100 ml Millipore water (mH<sub>2</sub>O). Glycerol forms a protective layer around the pRBC. Freezing medium was added to pRBC and transferred to cryotubes which were stored in liquid nitrogen.

Parasites were thawed using three thawing solutions. Solution A contains 12% sodium chloride (NaCl), solution B contains 1.8% NaCl, and solution C contains 0.9% NaCl plus 0.2% glucose. Solutions A and B were added sequentially, centrifuged (400 rcf) for 5 minutes and the supernatant removed. Solution C was added to the pellet and allowed to stand for 5 minutes. This mixture was centrifuged and the pRBC was cultured as described above.

#### 7.6.4 Preparation of reagents for developing plates

Malstat contains 400 µl Triton 100 which was heated gently to dissolve, 4 g L-lactate and 1.32 g Tris buffer in 200 ml of mH<sub>2</sub>O. This mixture was stirred to dissolve and allowed to cool. Then 22 mg of 3-acetyl pyridine adenine dinucleotide (APAD) was added and the pH adjusted to 9. Malstat is used to disrupt the red blood cell membrane thus exposing the parasite to nitro blue tetrazolium (Sigma). Nitro blue tetrazolium (NBT) solution consists of 160 mg NBT and 8mg phenazine ethosulphate in 100 ml of mH<sub>2</sub>O. All reagents were stored at 4°C.

#### 7.6.5 Antiplasmodial assay

Quantitative assessment of anti-malarial activity *in vitro* was determined by a modified method described by Makler (1993). This method of detection has proven to be reliable (Basco et.al., 1995). This colorimetric enzymatic assay is based on LDH of the parasite being distinguishable from host LDH activity using APAD as an analogue of nicotinamide adenine dinucleotide (NAD). Lactate is converted to pyruvate by pLDH using APAD as a coenzyme. In the presence of reduced APADH the yellow coloured NBT is converted to a purple formazan salt. The percentage parasite survival will be determined measuring the conversion of NBT by *P. falciparum*. Malstat is used to disrupt the red blood cell membranes thus exposing the parasite to NBT.

#### 7.6.6 Experimental set-up

Dose response experiments: A 96 well microtitre plate (Greiner Bio-One) was used for this assay. Samples were tested in duplicate on at least two separate occasions. The final hematocrit and parasitemia was 1% for all experiments. The first row was used as a blank containing 100 µl of the hematocrit and 100 µl complete medium (CM). The second row was used as a positive control containing 100 µl of the parasitemia and 100 µl CM. The remaining rows contained 100 µl of various drug concentrations in CM and 100 µl parasitemia. The starting concentration of samples tested was 100 µg/ml, which were serially diluted with two-fold dilutions in CM to give ten concentrations, the lowest being 0.195 µg/ml. Chloroquine (CQ) was tested

at an initial concentration of 100 ng/ml against the CQ sensitive strain and 1000 ng/ml against the CQ resistant strain of *P. falciparum*. The same dilution technique was used for all test samples. The microtitre plate was covered with a lid (Greiner Bio-One) and placed in a chamber. The chamber was gassed for about 2 minutes and incubated for 48 hours.

Three-point concentration test: The same experimental conditions were applied as for dose response test (described above). The plate set-up was adjusted to three concentrations, instead of ten as for a full dose response experiment. The concentrations were selected based on previous activity. CQ was tested at 30 ng/ml, 15 ng/ml and 7.5 ng/ml. Samples were tested in duplicate in two or three independent experiments.

#### **7.6.7 Developing plates**

After 48 hours the plate was re-suspended and 15µl of the suspension was transferred to corresponding wells in another plate containing 100µl Malstat. Then 25µl of NBT was added to each well. Air bubbles are removed with a hair dryer. The NBT is light sensitive thus the plate is placed in a dark cupboard to develop. The plate was read using a micro plate reader to measure the absorbance at 620 nm.

### **7.7 *In vitro* cytotoxicity testing**

#### **7.7.1 Preparation of medium for cell culture**

Dulbecos Modified Eagles Medium (DMEM) (Highveld Biological, South Africa) consists of 13.53 g/L DMEM and 3.7 g/L NaHCO<sub>3</sub> and pH 7.1. Nutrient mixture F-12 HAM (HAMS) (Sigma) contains 10.7 g/L HAMS and 1.176 g/L NaHCO<sub>3</sub> and the pH adjusted to 7.1. Foetal calf serum (Highveld Biological, South Africa) is heat inactivated and stored at -20°C. Cells were maintained in CM containing DMEM, HAMS and gentamycin (500 µl/L or 0.05 g/L) supplemented with 10% heat inactivated foetal calf serum (FCS) (Highveld Biological, South Africa). The prepared

reagents underwent the same filtering technique as for parasite culturing reagents (8.5.1). Reagents and medium were stored at 4°C.

### **7.7.2 Sub culturing cells**

Cell culture was done under sterile conditions. The cells were maintained as monolayers in culture flasks (vented cap) (Greiner Bio-One) and incubated at 37°C in a 5% CO<sub>2</sub> humidified atmosphere. Once cells were confluent the medium was removed and the cells rinsed twice with 10ml of sterile phosphate buffer saline (PBS). Then 5 ml of 10% sterile trypsin (Highveld Biological, South Africa) was added. This procedure is done quickly as too long exposure to trypsin will denature the cell membrane. To inactivate the trypsin 5 ml CM was added. This cell suspension was centrifuged at 750 rpm for 5 minutes. The supernatant was poured off and the pellet was re-suspended in 5 ml CM. This cell mixture was used for the assay and freezing. Approximately 1 ml of the cell suspension was transferred to a new flask containing 9ml of CM.

### **7.7.3 Cryostorage and thawing of cells**

The freezing medium used contains 90% FCS and 10% DMSO. The cell suspension was mixed with freezing medium and stored in liquid nitrogen.

Thawing medium consists of 30% FCS, 35% DMEM and 35% HAMS. The frozen cells were thawed and added to cold thawing medium. This mixture was centrifuged at 750 rpm for 5 minutes and the supernatant poured off. The pellet was re-suspended in thawing medium and transferred to a flask containing 9ml of thawing medium. The flask was incubated at 37°C (5% CO<sub>2</sub>).

### **7.7.4 Cytotoxicity assay**

The mammalian CHO cell line was used for cytotoxicity testing and was obtained from S.Schwager, Department of Medical Biochemistry, University of Cape Town, South Africa.

Cytotoxicity testing is important to establish whether the activity of the sample is selective to the test organism and not toxic to cells. This cell-line is easy to grow and widely used for cytotoxicity testing. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) assay was first described for use as a rapid colorimetric assay for cellular growth and survival, and compares well with other available assays (Mosmann et al., 1983 and Rubinstein et al., 1990). The tetrazolium salt MTT was used to measure growth and chemosensitivity. The tetrazolium ring is cleaved in active mitochondria thus only viable cells are able to reduce the water-soluble yellow coloured MTT to water-insoluble purple coloured formazan (Sieuwert et al., 1995).

#### **7.7.5 Experimental set-up**

Sample preparations were the same as for antiplasmodial screening (7.5). Each sample was tested in triplicate in two or three independent experiments. A 96 well microtitre plate (Greiner-Bio One) was used. The initial concentration of test samples was 100 µg/ml, which were serially diluted in complete medium with 10-fold dilutions to give 5/6 concentrations, the lowest being 0.001 µg/ml or 0.0001 µg/ml. Cells were sub cultured as previously described (7.7.2). A small aliquot of the cell suspension was mixed with 1% crystal violet for counting. The concentration of cells used was  $10^5$  cells/ml.

Plating cells: Row H was used as a blank and contained 200 µl of CM. Rows G to A contained 100µl of CM and 100µl of the cell suspension. The plates were incubated for 24 hours.

Addition of test samples: After 24 hours the medium was removed. Row G was used as a positive control which contained cells and CM. Row F to A contained dilutions of the test samples and cells. The final volume in each well was 200µl. The plate was covered with a lid (Greiner Bio-One) and incubated at 37°C (5% CO<sub>2</sub>) for 48 hours.

### 7.7.6 Developing plates

After 48 hours 25 µl of MTT was added to each well and incubated. After 4 hours the plate was centrifuged at 200 rpm for 10 minutes. The medium was removed and 100 µl DMSO was added to dissolve the formazan crystals. The formazan product was determined using a microplate reader at 540nm wavelength.

### 7.8 Data analysis for antiplasmodial and cytotoxicity assay

Data was analysed in Microsoft Excel® 2002. The IC<sub>50</sub>-value is defined as the concentration inhibiting 50% of growth and was determined using a non-linear dose response curve fitting analysis in GraphPad Prism v.4.0. IC<sub>50</sub>-values are given as a mean value with standard deviations.

### 7.9 *In vitro* antimycobacterial, antibacterial and antifungal testing

Testing was performed by Tracy Seaman in the Division of Pharmacology (University of Cape Town).

#### 7.9.1 Antimycobacterial assay

##### 7.9.1.1 Broth micro dilution method

*M. aurum* A+ was first isolated from human sputum (Tsukamura et al., 1966) and was obtained from the Pasteur Institute Culture collection (CIP). Middelbrook 7H9 broth (Becton Dickinson, Sparks, MD, USA) supplemented with 10% OADC (oleic acid, albumin, dextrose and catalase) (Remel, Lenexa, KS, USA) was used to grow *M. aurum* A+ (CIP 104482) from glycerol stocks stored at -70°C.

Experimentation purposes: Middelbrook 7H9 broth was inoculated with a colony of growth and allowed to grow overnight at 37°C. Experiments were performed in 96-

well micro titre plates. The high-throughput screening of antimycobacterial agents in combination with the colorimetric method using tetrazolium salts was used (Chung et al., 1995 and Eloff et al., 1998).

Each test sample was tested in triplicate. Row A was used as a blank which contained 100 µl sterile distilled water (dH<sub>2</sub>O) and 100 µl 7H9 broth. Rows C – H were used to prepare 2-fold dilutions of the test sample in sterile dH<sub>2</sub>O to give ten concentrations. The culture was adjusted to an optical density of 0.125 at 550 nm to yield approximately  $1 \times 10^5$  cfu/ml. Then 100 µl of this dilute culture was added to all wells except the blank row. To prevent dehydration the plates were loosely sealed in plastic bags and incubated (37°C) for 72 hours.

Developing plates and data analysis: Growth was measured using p-iodonitrotetrazolium salt (INT, Fluka) prepared to 0.4 mg/ml solution. INT changes colour to red in the presence of metabolising organism (Eloff et al., 1998). After incubation 40 µl of INT was added to the plates. Plates were incubated overnight at 37°C. The lowest concentration with no indication of red as a result of INT was regarded as the MIC. IC<sub>50</sub>-values were determined with a Modulus micro plate reader at 600 nm. Data was analysed in Microsoft Excel® 2002 and dose response curves were plotted using a non-linear dose response curve fitting analysis in GraphPad Prism v.4.0.

#### **7.9.1.2 BACTEC method**

Testing against *M. tuberculosis* was performed under biologically safe conditions according to the methodology described by Lall and Meyer (1999) for the antimycobacterial testing of crude plant extracts. Susceptibility testing against *M. tuberculosis* H37Rv ATCC 25177 was performed using the radiometric BACTEC 460 system (Becton Dickinson). This system monitors the production of radiolabelled CO<sub>2</sub> by the metabolizing organism which indicates the effect of the test sample on organism viability.



The BACTEC 12B vial used for this experiment contains radiolabelled  $^{14}\text{C}$ Carbon substrate which is taken up by the test organism. A glycerol stock of *M. tuberculosis* stored at  $-70^{\circ}\text{C}$  was used to inoculate BACTEC 12B vials. The vial was incubated at  $37^{\circ}\text{C}$  until the Growth Index (GI) reading of 999 was reached. Then 100  $\mu\text{l}$  of this culture suspension was used to inoculate a second BACTEC 12B vial which was incubated ( $37^{\circ}\text{C}$ ) until the GI reading was between 500 and 700.

Experimental set-up: The BACTEC 12B vials were incubated at  $37^{\circ}\text{C}$  for all experimentation purposes. Test vials were inoculated with 100  $\mu\text{l}$  of culture suspension from the second vial (GI = 500 – 700). Stock solutions of the test samples were prepared to 10.5 mg/ml in 50% methanol. The test samples (100  $\mu\text{l}$ ) were added to the test vials in duplicate. A three-point concentration test at 62.5, 125 and 250  $\mu\text{g/ml}$  was performed. A solvent control and an antibiotic, rifampicin (Sigma) at 2  $\mu\text{g/ml}$  were included in the experiment. Two culture control vials were also included in the experiment. A sample-free control vial was inoculated with 100  $\mu\text{l}$  of the culture suspension (GI = 500 – 700). Another sample-free control vial was inoculated with a 1:100 dilution of the organism in TB diluting fluid (NHLS Media department, Cape Town, South Africa). This control served as a representation of the bacterial population (1%) which is present in the test vials. GI readings of the test vials were read daily and compared to the 1:100 dilution control vial. The experiment was stopped if the GI readings in the 1:100 dilution control vial had progressed to two readings over a GI of 30.

Data analysis: The delta GI ( $\Delta\text{GI}$ ) was defined as the difference in GI values of the last two days. The  $\Delta\text{GI}$  values of the vials containing the test samples were determined. If the  $\Delta\text{GI}$  value of the test sample is less than that of the 1:100 controls, the bacterial population was considered susceptible to the test sample at that concentration. The MIC of a sample is defined as the lowest concentration inhibiting more than 99% of bacterial growth. If inhibition is observed at 250  $\mu\text{g/ml}$  but not at 125  $\mu\text{g/ml}$  then the MIC of the sample is recorded as 250  $\mu\text{g/ml}$ .

### 7.9.2 Antibacterial and antifungal assay

Stock cultures were obtained from the American Type Culture Collection (ATCC) of *C. albicans* ATCC 90028, *S. aureus* ATCC 12600 and *K. pneumoniae* ATCC 13883. The stock cultures were frozen by adding 20% sterile glycerol and storing at -70°C over long periods. Glycerol culture stocks which were used for experiments were stored at -20°C.

For experimentation against the fungus, *C. albicans*, sabouraud dextrose agar (SDA, Merck) plate was inoculated with a single glycerol stock of the organism and incubated at 30°C for 48 hours. The plate was loosely sealed in a plastic bag to prevent dehydration. After incubation 5ml RPMI (BioWhittaker) at pH 7.0 was inoculated with a single colony and incubated further overnight.

For experimentation against bacteria Gram positive *Staphylococcus aureus* and Gram negative *K. pneumoniae* tryptone soya agar (TSA, Oxoid) plates were inoculated with a single glycerol stock of either organism. Plates were loosely sealed in a plastic bag to prevent dehydration and allowed to grow overnight at 37°C. Then a single colony was used to inoculate 5 ml of tryptone soya broth (TSB, Oxoid) which was incubated at 37°C.

Broth micro-dilution method for *C. albicans*: A modified colorimetric method described by Clancy and Nguyen (1997) and Pfaller and Barry (1994) was used to measure growth. Experiments were performed under sterile conditions in 96-well micro titre plates. The final concentration of RPMI in the wells was 1 x RPMI. Plates were loosely sealed in plastic bags to prevent dehydration when incubated.

Experimental set-up: Test samples were prepared to 50 mg/ml in 100% MeOH and then diluted in sterile dH<sub>2</sub>O to a concentration of 1 mg/ml. A stock concentration of 10µg/ml of ciprofloxacin (Sigma) was used as the bacterial reference drug control and 25µg/ml of nystatin (Sigma) was used as the fungal reference drug control. Each test sample was tested in duplicate in two separate assays (total replicates = 4). Row A was used as a blank which contained 50 µl sterile dH<sub>2</sub>O and 50 µl RPMI for fungi.

RPMI was substituted with TSB for bacteria. Rows C – H were used to prepare 2-fold dilutions of the test sample in sterile dH<sub>2</sub>O to give concentrations ranging from 0.97 – 250 µg/ml.

Fungi: The overnight culture was diluted (1:50) in RPMI and 50 µl of mixture was added to all wells except the blank row to yield approximately  $1 \times 10^5$  cfu/well. Plates were incubated at 30°C for 48 hours.

Bacteria: The overnight culture was diluted (1:200) in TSB and 50 µl of mixture was added to all wells except the blank row to yield approximately  $1 \times 10^5$  cfu/well. Plates were incubated overnight at 37°C.

Developing plates: After incubation 40 µl of INT (0.4 mg/ml) was added to the plates. Plates were incubated at 30°C for 6 – 8 hours when tested against fungi and developed for 3 – 4 hours at either 37°C or room temperature when tested against bacteria. The lowest concentration with no indication of red as a result of INT was regarded as the MIC.

## **7.10 *In vivo* antiplasmodial testing**

### **7.10.1 Test animals**

The animals used were wild strains of C57 BL6 mice (7-10 weeks old) which were obtained from the animal unit at the University of Cape Town (South Africa). This study was approved by the Animal Research Ethics Committee (University of Cape Town). Animal handling complied with guidelines followed by the University of Cape Town which were adapted from the South African Medical Research Council (MRC, 2004). The animals were housed in standard cages at room temperature (24°C). Their diet was standard pellets and water which were freely available.

### 7.10.2 Infection

The cryopreserved CQR *Plasmodium yoelii* NS strain originated from the Institute for Evolutionary Biology, Edinburgh, Scotland, United Kingdom. The cryopreserved CQS *Plasmodium berghei* ANKA strain originated from the Swiss Tropical Institute, Basel, Switzerland. Stocks were kept in liquid nitrogen at -80°C. The cryopreserved parasite stocks were thawed and two mice were interperitoneally infected. The parasitemia was allowed to develop over 5 days. Blood was collected by an incision at the tail vein and the parasitemia was determined microscopically using 10% giemsa stained thin blood smears. The cells/ml was determined using a hemacytometer and diluted with phosphate buffered saline (PBS) to a suspension of  $5 \times 10^6$  parasitized cells/ml. The animals were infected with 0.2 ml of  $1 \times 10^6$  parasitized cells/ml solution. Percentage growth inhibition was determined as follows:

$$\% \text{ Growth inhibition} = \frac{\text{parasitemia of control} - \text{parasitemia of test sample}}{\text{parasitemia of control}} \times 100$$

### 7.10.3 Dose preparation

CQ was used as the reference drug in all experiments and was prepared to 15 mg/kg bw in Millipore water. Plant extracts were prepared to 400 mg/kg bw or 800 mg/kg bw in 5% sodium bicarbonate (with or without 5% ethanol) or a lipid-based formulation. Compound 3 was tested at 100 mg/kg bw and was dissolved in 10% DMSO. Test samples were administered as a suspension if not completely dissolved. Solvent concentrations for 10% DMSO, 5% sodium bicarbonate and the lipid-based formulation have proven to be safe *in vivo* (Peters et al., 2002 and Wu et al., 2006). Controls for dose preparations were performed using the 4-day suppressive test (Fig 16). The CQ control group and all test groups except the control were treated daily. The route of administration was by gavage for all experiments. The mice received 200  $\mu$ l of the test suspensions except for the SMEDDS formulation volumes which varied depending on the dose and body weight. The mice were monitored daily and the weights were determined.

### **7.10.3 Schizontocidal activity**

#### **7.10.3.1 Four-day suppressive test**

The mice were tail-bled on days 3, 4 and 7 post infection to determine the parasitemia using thin blood smears. The 4-day suppressive test was followed for this experiment similar to the test described by Peters et al. (1993). Treatment was started 24 hours post infection. The positive control group did not receive treatment during the study. Parasitemia is given as a mean value with standard deviations. The mice were sacrificed 7-9 days post infection.

#### **7.10.3.2 Seven-day suppressive test**

The mice were tail-bled on days 4 and 7 post infection to determine the parasitemia using thin blood smears. Mice were grouped into 4 groups with 2 groups of 5 mice for the test samples and 2 groups of 3 mice for the controls (CQ and positive). Treatment was started 24 hours post infection. The positive control group did not receive treatment during the study. Parasitemia is given as a mean value with standard deviations. The mice were sacrificed 9-10 days post infection.

### **7.10.4 Parasite clearance experiment**

The clearance experiment was performed according to a modified method described by Hou et al. (2004). Mice were grouped into 4 groups with 2 groups of 5 mice for the test samples and 2 groups of 4 mice for the controls (CQ and positive). Parasitemia was allowed to develop to 5 – 10% before commencing treatment. The crude extract was administered orally at two different doses for 4 consecutive days. The positive control group did not receive treatment during the study. The mice were tail-bled on days 4, 7 and 8 post infection to determine the parasitemia using thin blood smears. Parasitemia is given as a mean value with standard deviations. The mice were sacrificed 7-9 days post infection.

### **7.10.5 Prophylactic experiment**

The prophylactic experiment was performed according to a modified method described by Rane et al. (1979). Treatment was started four days prior to infection for 4 consecutive days. The treatment was stopped on day 0 (day of infection). The parasitemia was monitored from day 4 post infection. Mice were grouped into 4 groups with 2 groups of 5 mice for the test samples and 2 groups of 4 mice for the controls (CQ and positive). The positive control group did not receive treatment during the study. Parasitemia is given as a mean value with standard deviations. The mice were sacrificed 8-9 days post infection.

## **7.11 Preliminary pharmacokinetic study of *S. aethiopicus* using LC-MS/MS technology**

### **7.11.1 Test animals**

Animal handling and conditions were the same as described previously (7.10.1). One batch of 10 mice was used for all experiments. A washout period of 2-3 weeks was allowed between experiments. O<sup>+</sup> human blood (Western Province Blood Transfusion Service, Groote Schuur Hospital, Cape Town, South Africa) was used for the preparation of standard controls. The routes of administration used were oral (gavage) or subcutaneous. A volume of 200 µl was given to mice except for the oil-based formulation which varied according to weight.

### **7.11.2 Method development for bioavailability study of compound 3**

#### **7.11.2.1 Mass spectrometry and chromatography conditions**

An Applied Biosystems 3200 Q-trap mass spectrometer coupled to a high performance liquid chromatography system was used to analyse the samples. A Phenomenex C<sub>18</sub> (15 cm x 4.6 mm, 5 µm) column with an injection volume of 5 µl was used for analyses. The MP used was ACN:0.1% formic acid (6:4) which was run

isocratically for 1.5 min. Stock solutions of compound 3 were prepared to 10 µg/ml in whole blood.

#### **7.11.2.2 Sample preparation**

Liquid-liquid extraction of compound 3: 10 µl of spiked whole blood (10 µg/ml) was added to 50 µl of the respective pH buffers. An organic solvent, ethyl acetate (5 x volume of pH buffer, 250 µl) was added. EA was selected as it was used for extraction of plant material. This mixture was vortexed for 1 minute, centrifuged at 13 000 rcf (5 min) and 200 µl of the supernatant removed. The supernatant was dried in MS-inserts using a cold trap system and reconstituted in 100 µl MP. This mixture was vortexed for 15 seconds and injected onto the LC-MS/MS for analysis.

Extraction of compound 3 in different matrixes: Approximately 1 ml of whole blood was separated into a RBC pellet and plasma by centrifugation (13 000 rcf for 5 min). Then 30 µl of plasma or RBC's were added to 150 µl pH 10 buffer. EA (750 µl) was added to the mixture, vortexed for 1 min and centrifuged. The supernatant was removed, dried in MS-inserts using a cold trap system and reconstituted in 100 µl MP for analysis. The percentage recovery was determined as a mean value (standard deviation):

$$\% \text{ Recovery} = \text{peak area of sample} / \text{peak area of standard control} \times 100$$

### **7.11.3 Bioavailability study of compound 3**

#### **7.11.3.1 Mass spectrometry conditions**

An Applied Biosystems 3200 Q-trap mass spectrometer coupled to a high performance liquid chromatography system using a Phenomenex C<sub>18</sub> (15 cm x 4.6 mm, 5 µm) column with an injection volume of 5 µl. The MP was acetonitrile (ACN):0.1% formic acid (6:4) and isocratic run rate of 1.5 min.

#### **7.11.3.2 Sample preparation and extraction process**

Standards of compound 3 were prepared to a stock solution of 1 µg/ml in MP. Stock solutions were serially diluted two-fold from 10 µg/ml to 0.15 µg/ml in plasma. Samples were stored at -20°C over short periods and at -80°C for long term storage.

Blood sampling and extraction of test samples: Blood was collected by an incision at the tail vein or the tip of tail, in heparin-coated eppendorfs and immediately placed on ice. Approximately 40 µl of blood was collected at 5 time intervals (Table 15). Blood samples were centrifuged at 13 000 rcf for 5 min and 10 µl of plasma removed. The plasma was added to 50 µl pH buffer and extracted immediately. 250 µl of EA was added to plasma and pH buffer mixture. This was vortexed for 1 min, centrifuged for 5 min at 13 000 rcf and 200 µl of the supernatant removed. The supernatant was dried in LCMS inserts using a cold trap system. The dried material was reconstituted the following day in 100 µl of MP for analysis.

#### **7.11.4 Bioavailability study of crude extract**

Experimental conditions were as described in 7.11.3.1. Standards of compound 1, 2 and 3 were prepared to a stock solution of 1 µg/ml in MP. Stock solutions were serially diluted two-fold from 10 µg/ml to 0.15 µg/ml in plasma. Samples were stored at -20°C over short periods and at -80°C for long term storage.

##### **7.11.4.1 Sample preparation and extraction process**

Standards of compound 3 were prepared to a stock solution of 1 µg/ml in MP. Stock solutions were serially diluted two-fold from 10 µg/ml to 0.15 µg/ml in plasma. Samples were stored at -20°C over short periods and at -80°C for long term storage.



### 7.11.5 Metabolite study of compound 3

#### 7.11.5.1 Mass spectrometry and chromatography conditions

An Applied Biosystems 3200 Q-trap mass spectrometer coupled to a high performance liquid chromatography system using a Phenomenex C<sub>18</sub> (15 cm x 4.6 mm, 5 µm) column with an injection volume of 5 µl. The MP was ACN:0.1% formic acid (6:4) and an isocratic run rate of 15 min. The molecular ion of compound 3 (*m/z* 244) and the major product ion of *m/z* 116 was selected. For the precursor experiment the LC-MS/MS the molecular ion was first isolated on the basis of its mass/charge ratio and then passed into the collision cell. The collision cell is then filled with an inert gas such as helium and a potential difference is applied. Product ions are formed when the energized ions collide with the gas molecules which move out of the collision cell and hit the detector. Product ions of unknown metabolites should correspond to the product ions of the parent compound.

#### 7.11.5.2 Sample preparation

Samples were collected over a period of two hours. Samples were pooled and extracted immediately after collection.

Blood sampling: Approximately 10 – 20 µl of blood was collected at various time intervals. 30 µl of whole blood was extracted with ACN, vortexed for 2 min and centrifuged. The supernatant was injected onto the LC-MS/MS.

Urine samples: Mice normally urinate by reflex when handled thus no added stress was placed on the animal to provoke urination. Approximately 10 µl of urine was collected at the same time as blood collections except for the collection after 1 hour which yielded too little material. The urine samples were pooled after collection and diluted in MP (1:50) for analysis.

Faeces samples: The cage of the test animal was cleaned 24 hours before the experiment for collection of droppings. Droppings were collected on the day of the

experiment before dosing which served as the blank control. Droppings were collected again 24 hours after the experiment. Both samples were extracted with 100 ml of ACN. This mixture was stirred for 30 min, centrifuged for 5 min at 10 000 rcf and the supernatant removed for analysis.

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# *Appendix I*

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## *Dose-response curves*

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## Dose-response curves

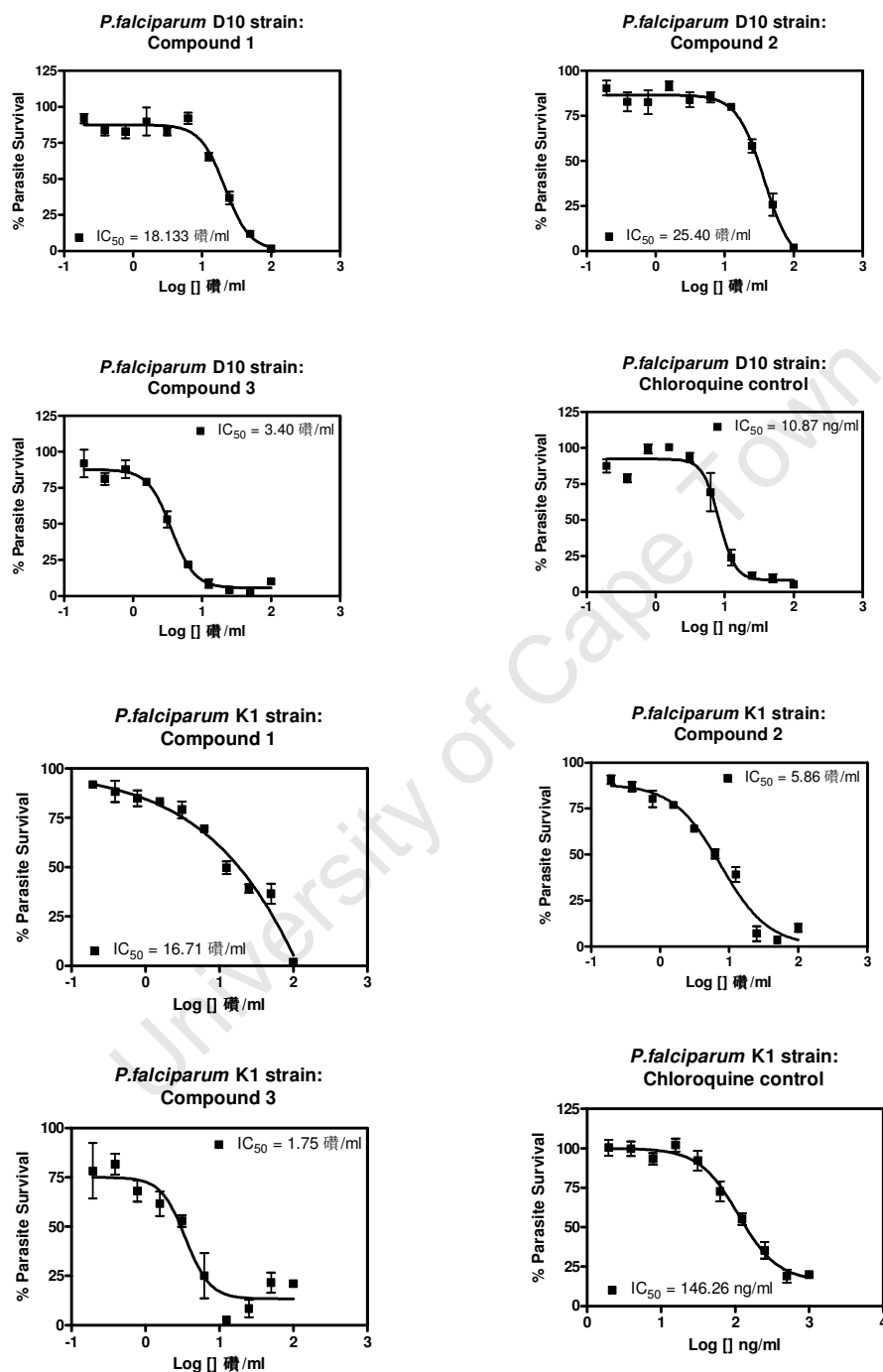


Figure 54 Pooled dose-response curves compounds isolated from *S. aethiopicus* against the CQS D10 strain and CQR K1 strain of *P. falciparum* (n = 2)

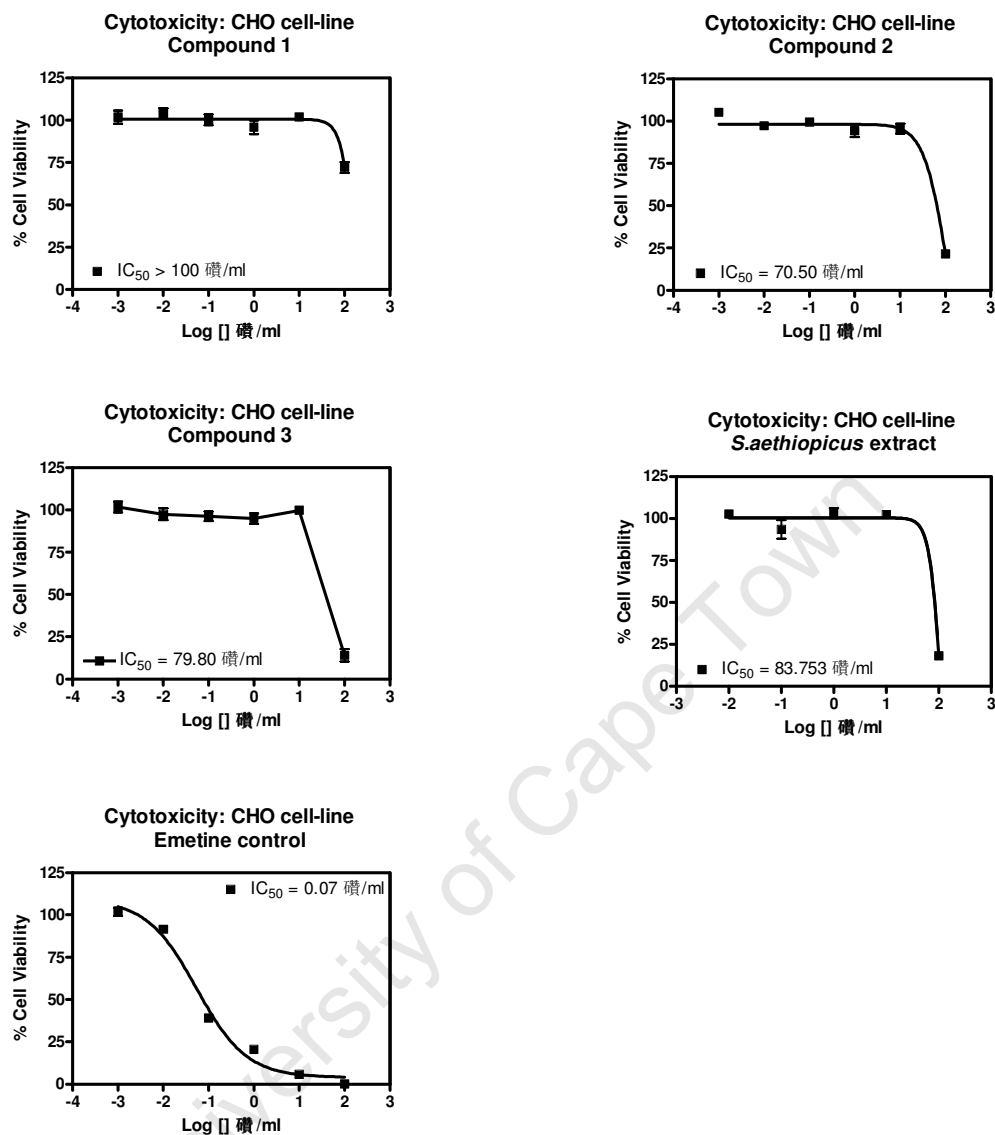


Figure 55 Pooled dose-response curves of crude EA extract and compounds isolated from *S. aethiopicus* against the CHO cell-line (n = 2)

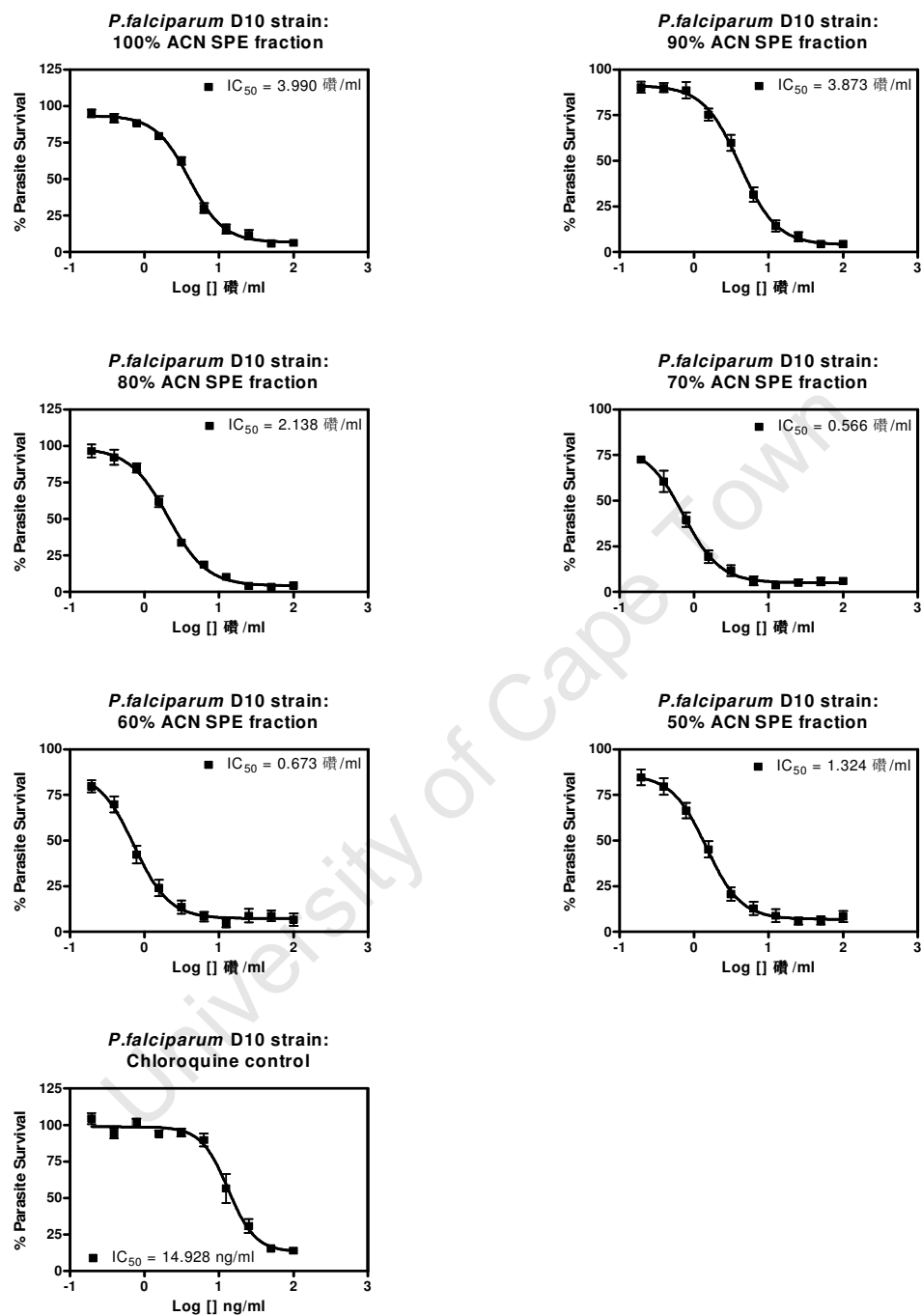


Figure 56 Pooled dose-response curves of SPE fractions from *S. aethiopicus* against CQS D10 strain of *P. falciparum* (n = 4)

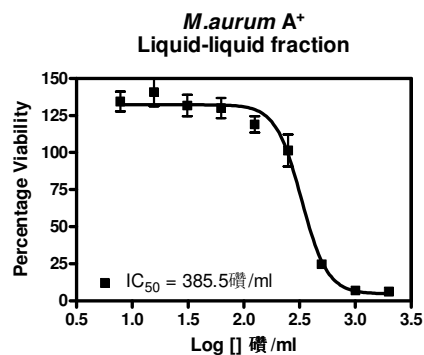


Figure 57 Dose-response curve of liquid-liquid fraction (pH 11) from *S. aethiopicus* extract against *M. aurum* A<sup>+</sup>

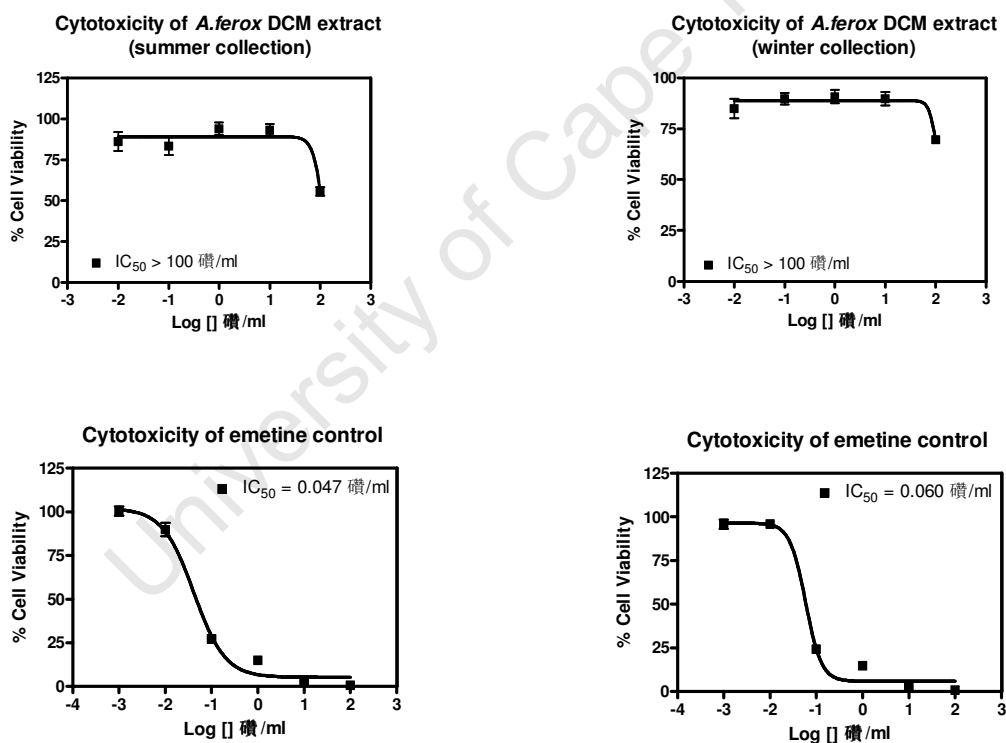


Figure 58 Pooled dose-response curves of *A. ferox* crude extract against the CHO cell-line (n = 2)



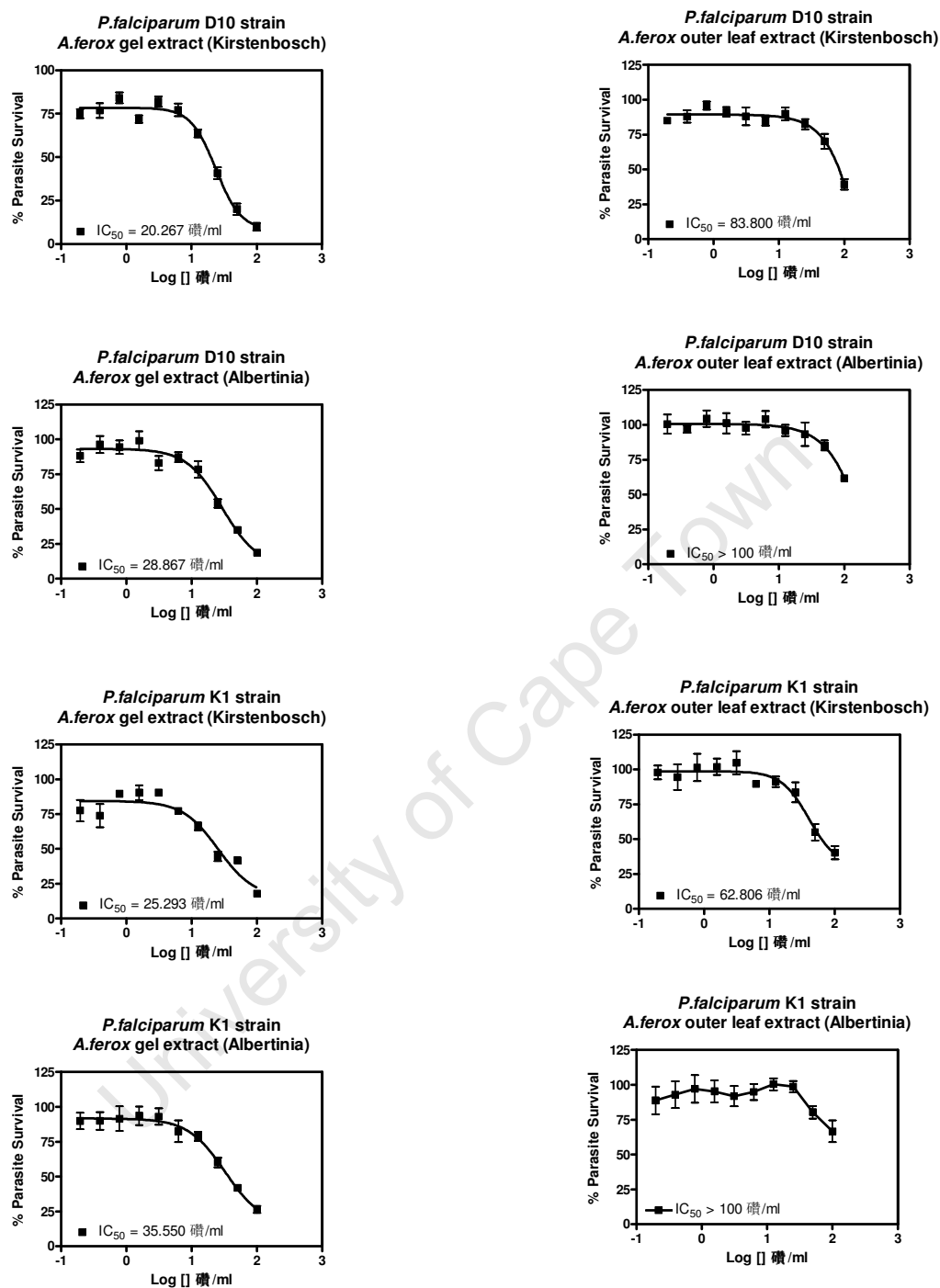


Figure 59 Pooled dose-response curves of *A. ferox* against CQS D10 strain and CQR K1 strain of *P. falciparum* ( $n = 2$ )

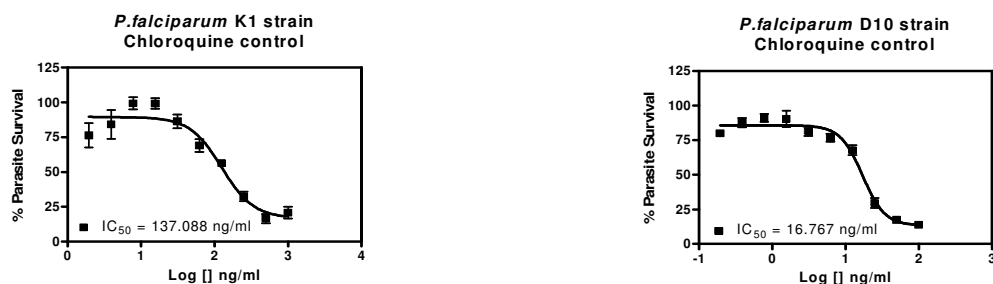


Figure 60 Pooled dose-response curves of chloroquine against the CQS D10 strain and CQR K1 strain of *P. falciparum* (n = 2)

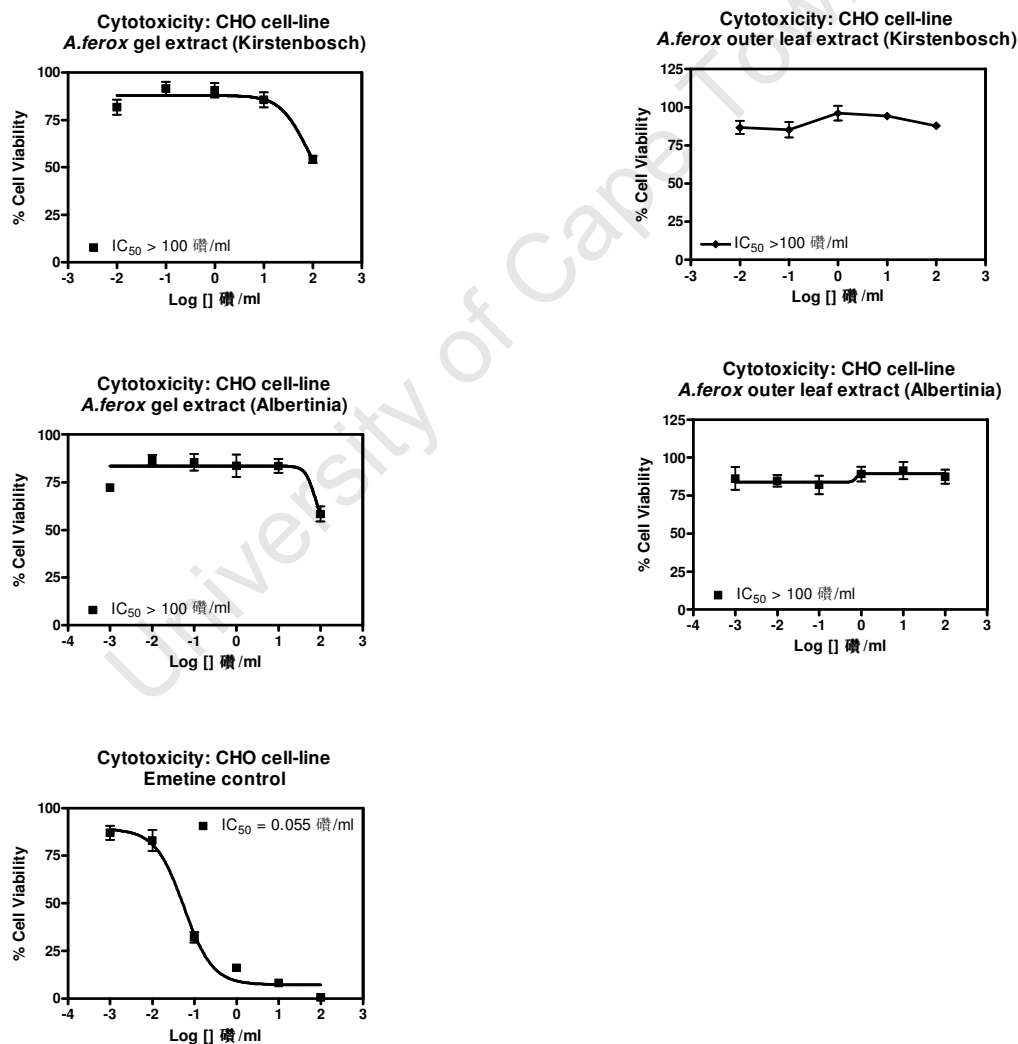


Figure 61 Pooled dose-response curves of *A. ferox* against the CHO cell-line (n = 2)

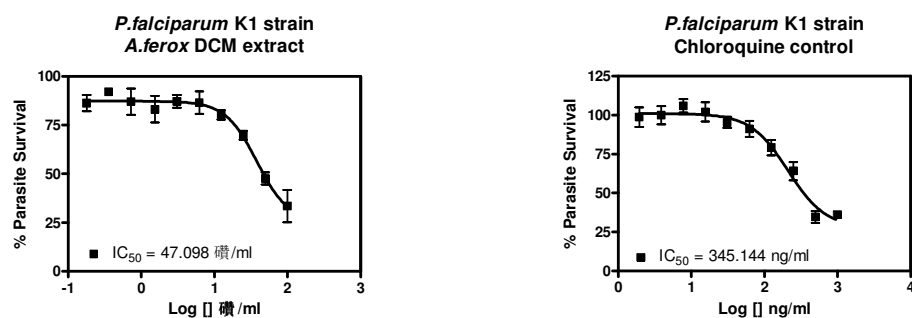


Figure 62 Pooled dose-response curves of *A. ferox* DCM extract (n = 2) collected in summer and chloroquine control (n = 3) against CQR K1 strain of *P. falciparum*

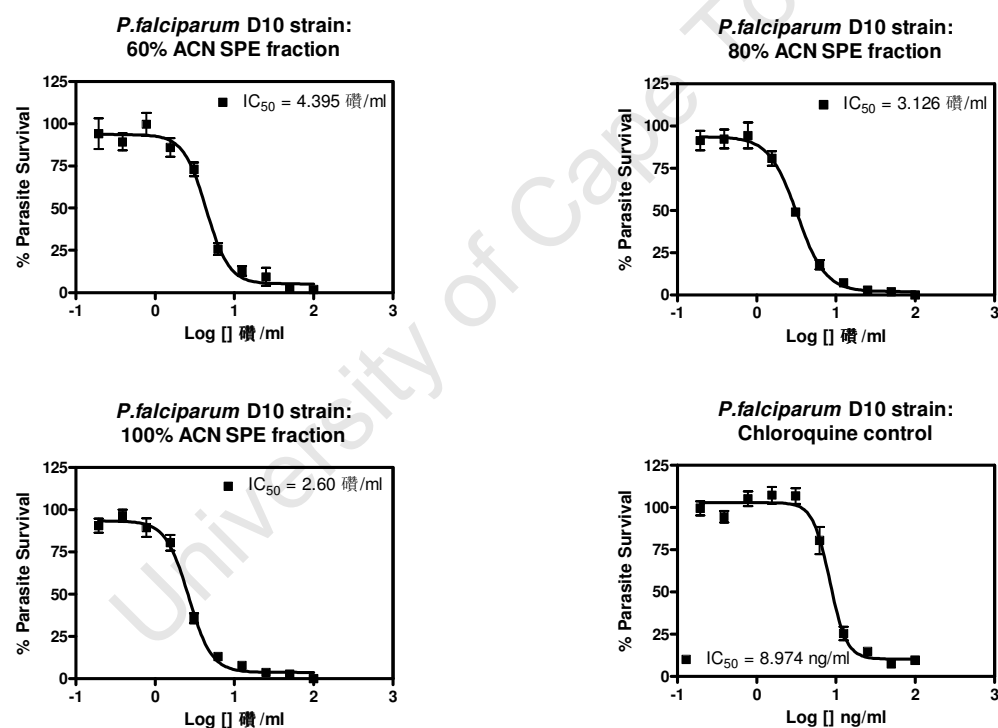


Figure 63 Pooled dose-response curves of SPE fractions (20% ACN increments) against *P. falciparum* CQS D10 strain

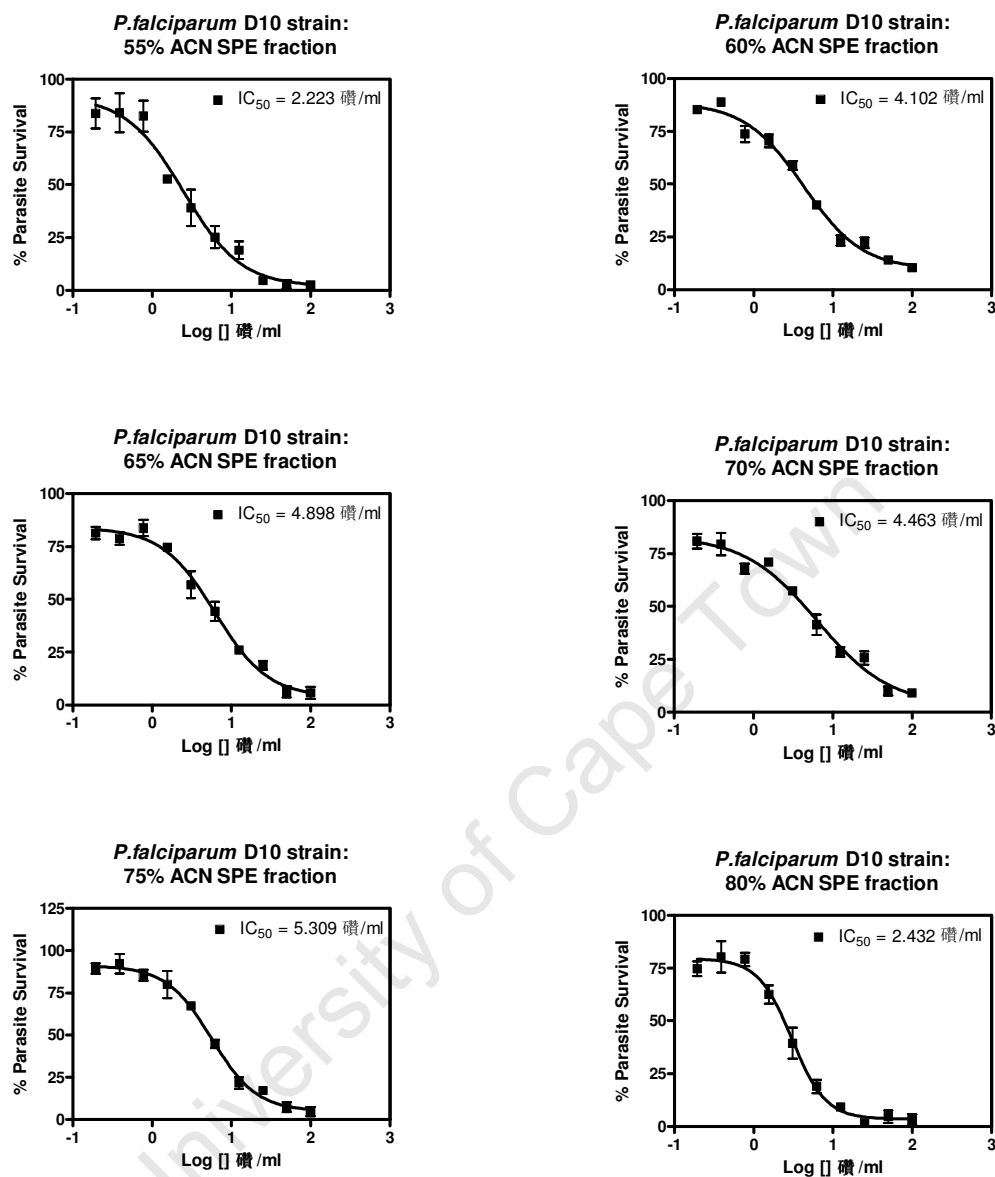


Figure 64 Pooled dose-response curves of SPE fractions (5% ACN increments) from *A. ferox* against CQS D10 strain of *P. falciparum* (n = 2 / 3)

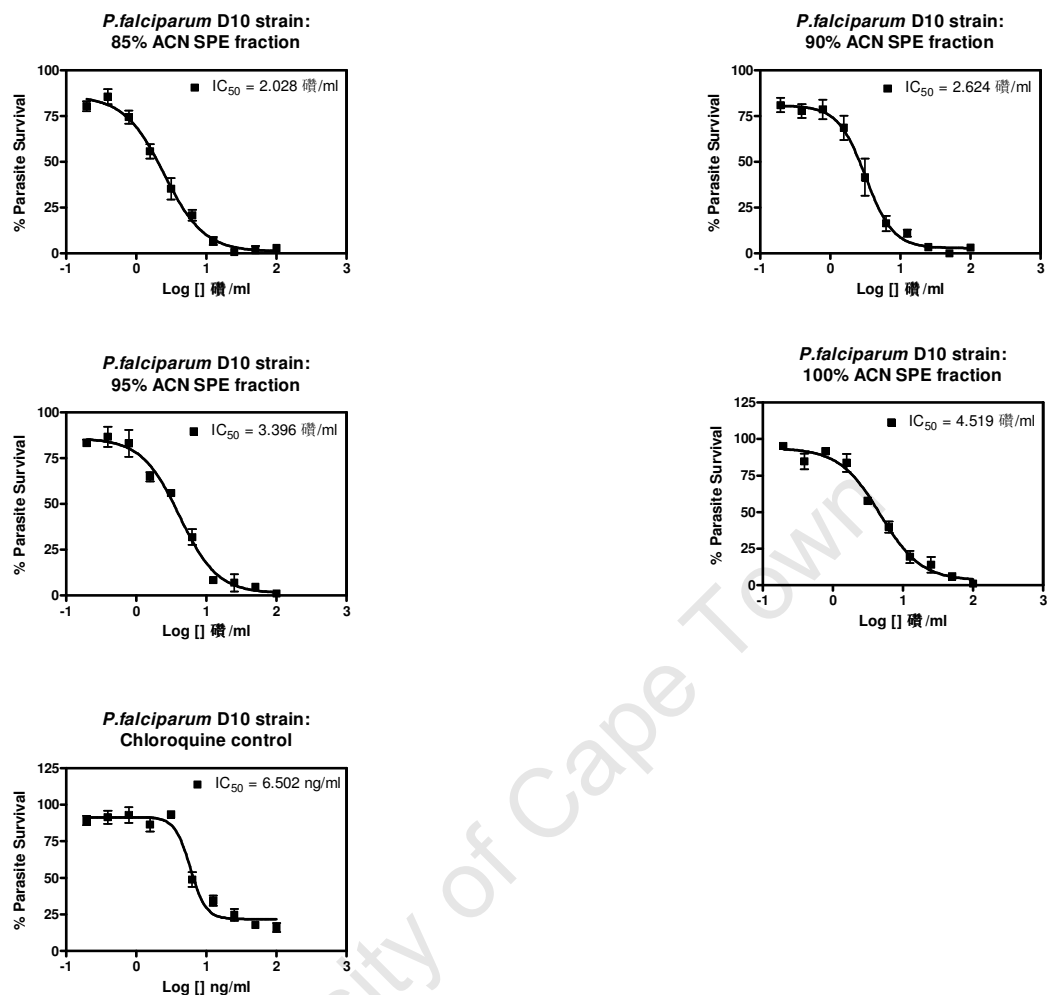


Figure 65 (Continued) Pooled dose-response curves of SPE fractions (5% increments) from *A. ferox* tested against CQS D10 strain of *P. falciparum* (n = 2 / 3)

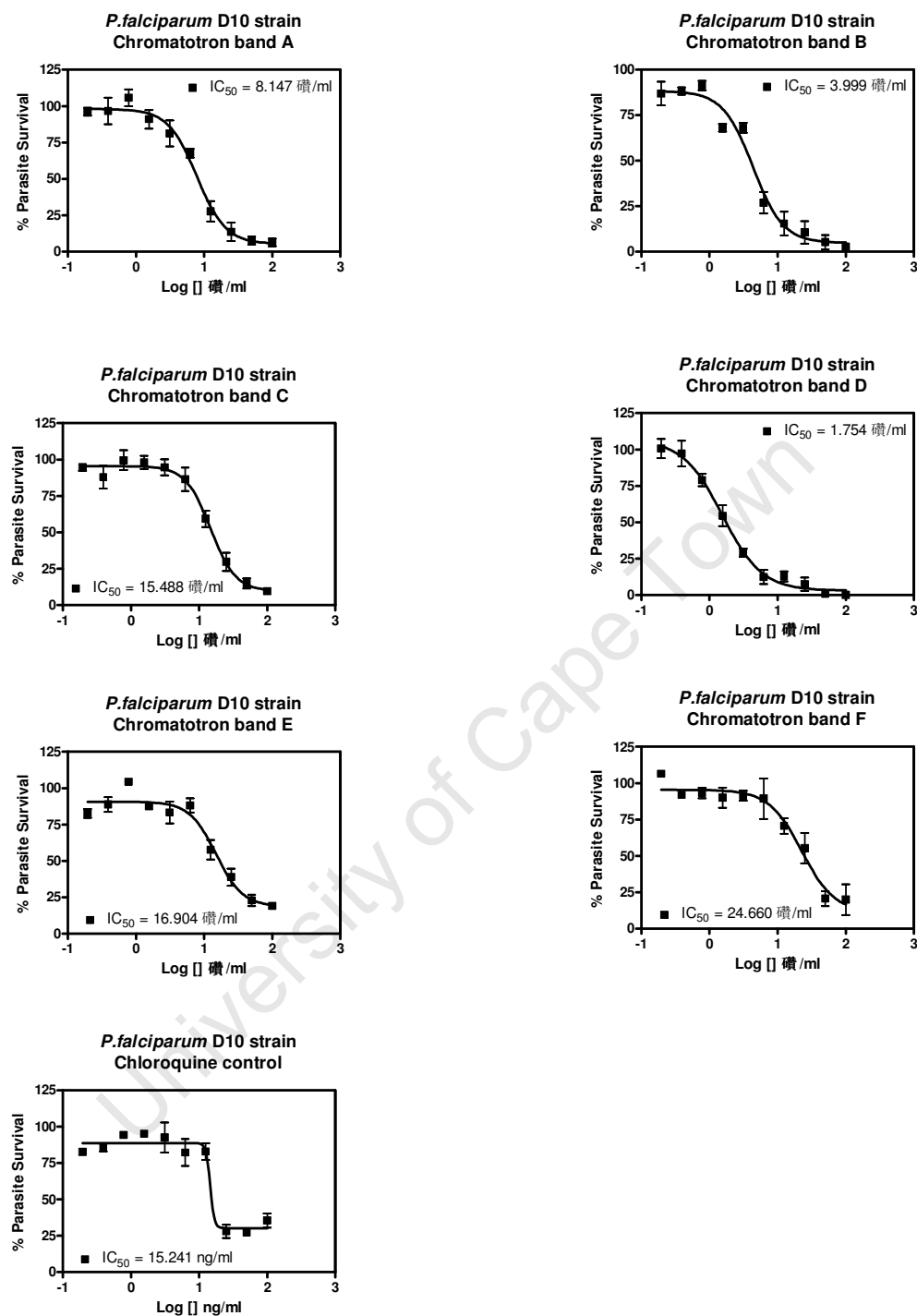


Figure 66 Pooled dose-response curves of chromatotron fractions obtained from 90% ACN SPE fraction tested against the CQS D10 strain of *P. falciparum* (n = 2)

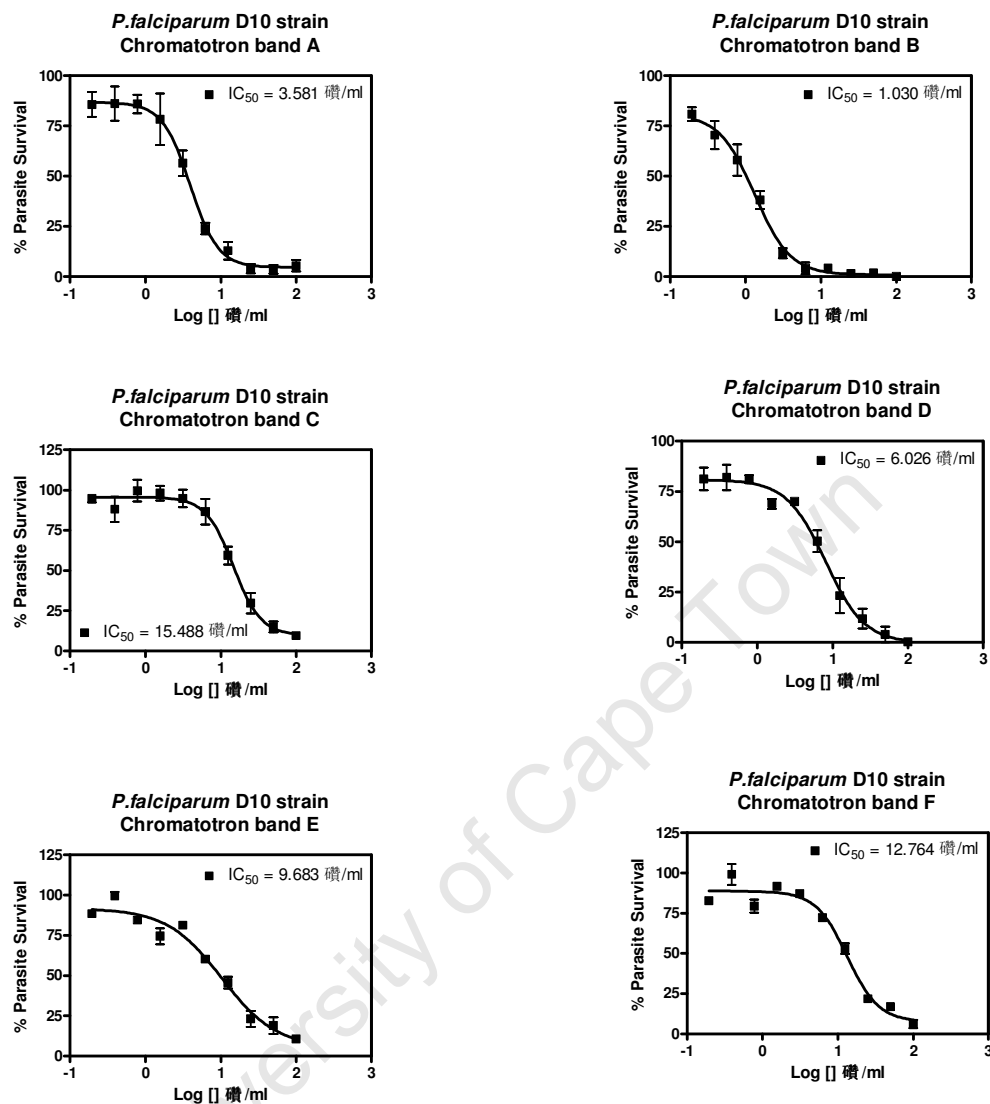


Figure 67 Pooled dose-response curves of chromatotron fractions obtained from 85% ACN SPE fraction tested against the CQS D10 strain of *P. falciparum* (n = 2)

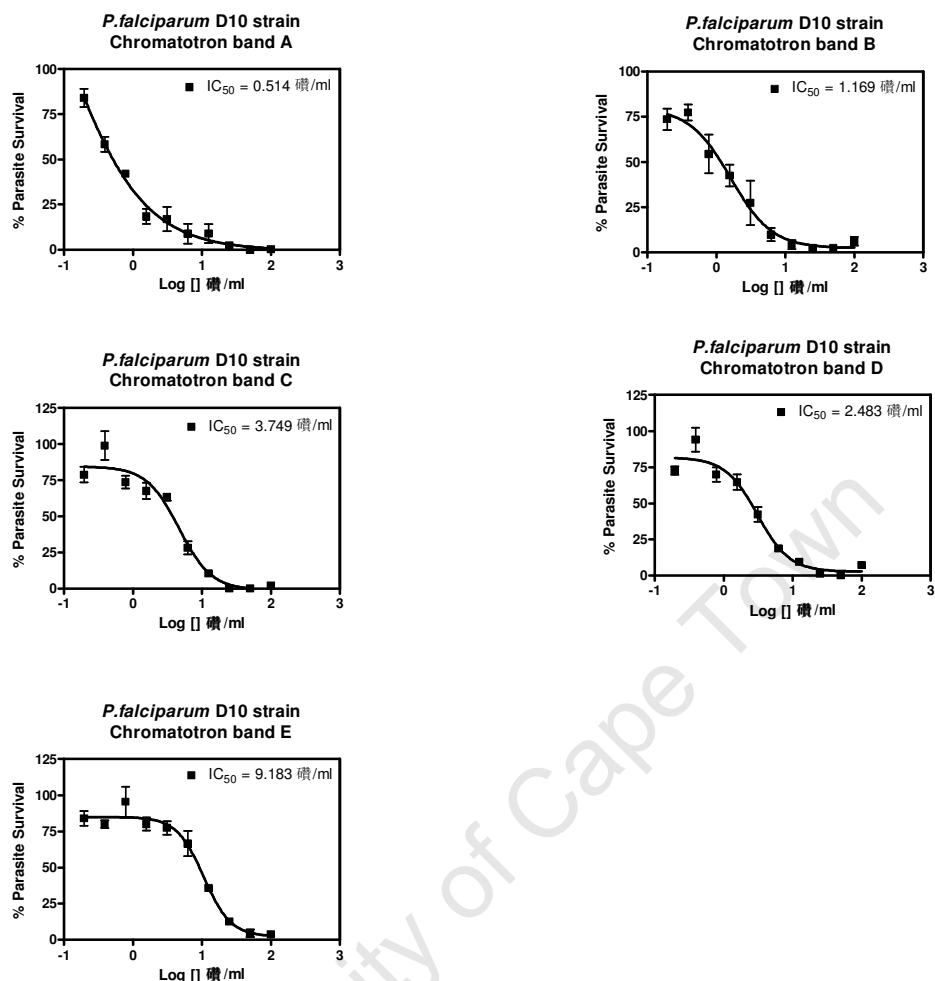


Figure 68 Pooled dose-response curves of chromatotron fractions obtained from 80% ACN SPE fraction tested against the CQS D10 strain of *P. falciparum* (n = 2)

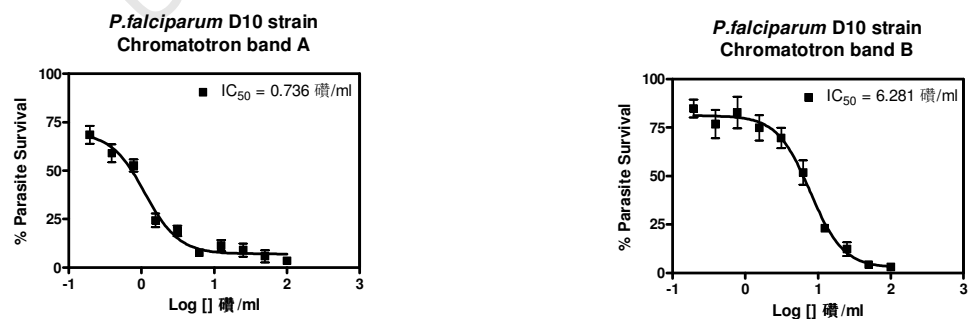


Figure 69 Pooled dose-response curves of chromatotron fractions obtained from 60% ACN SPE fraction tested against the CQS D10 strain of *P. falciparum* (n = 2)



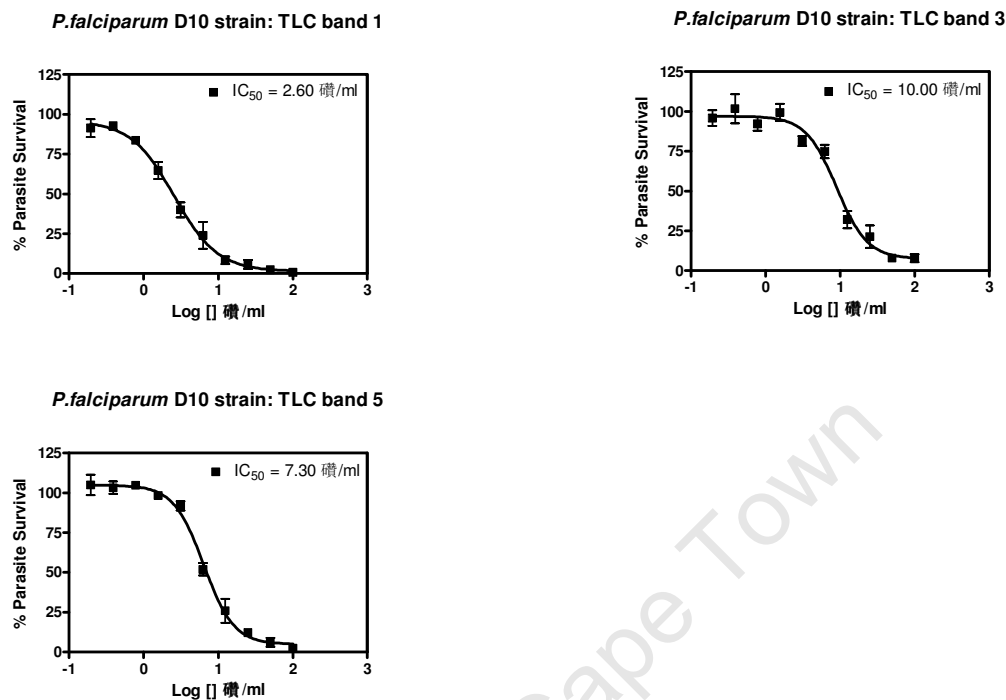


Figure 70 Pooled dose-response curves of TLC bands from chromatotron band D, 90% SPE ACN fraction against the CQS D10 strain of *P. falciparum* ( $n = 2$ )

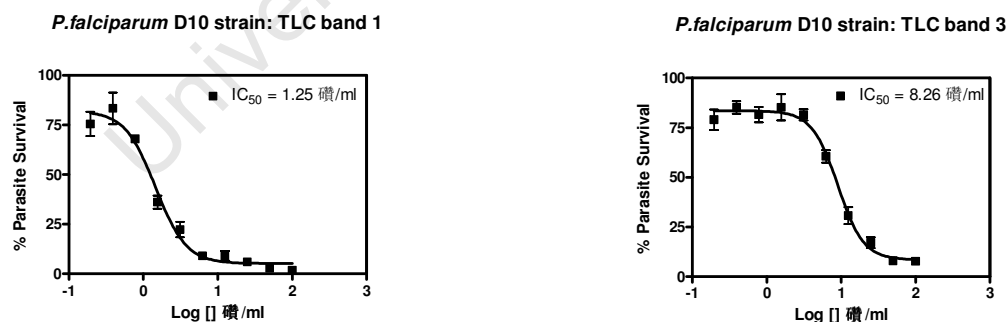


Figure 71 Pooled dose-response curves of TLC bands from chromatotron band A, 80% SPE ACN fraction against the CQS D10 strain of *P. falciparum* ( $n = 2$ )

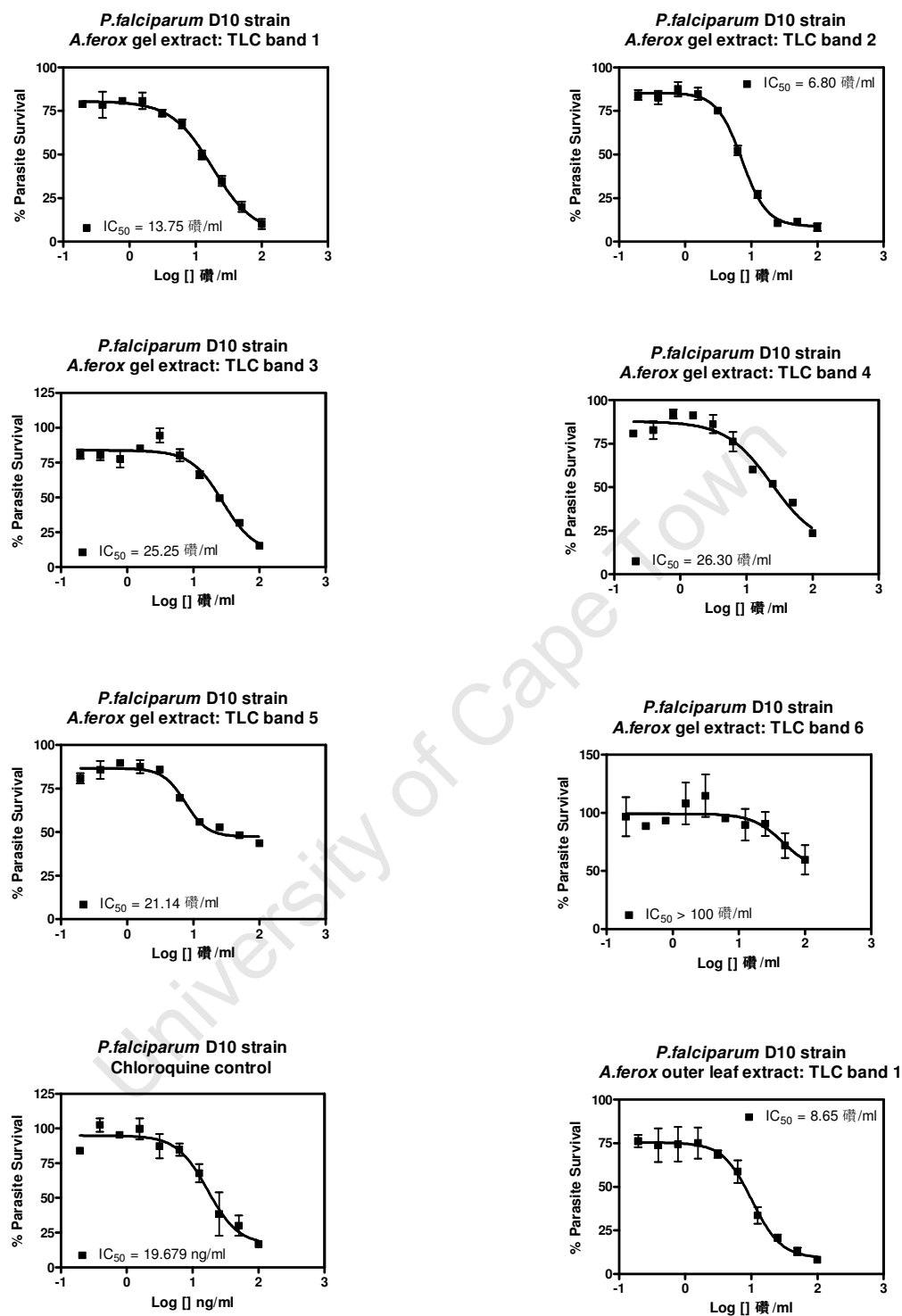


Figure 72 Pooled dose-response curves of *A. ferox* extracts separated via TLC tested against the CQS D10 strain of *P. falciparum* (n = 2)

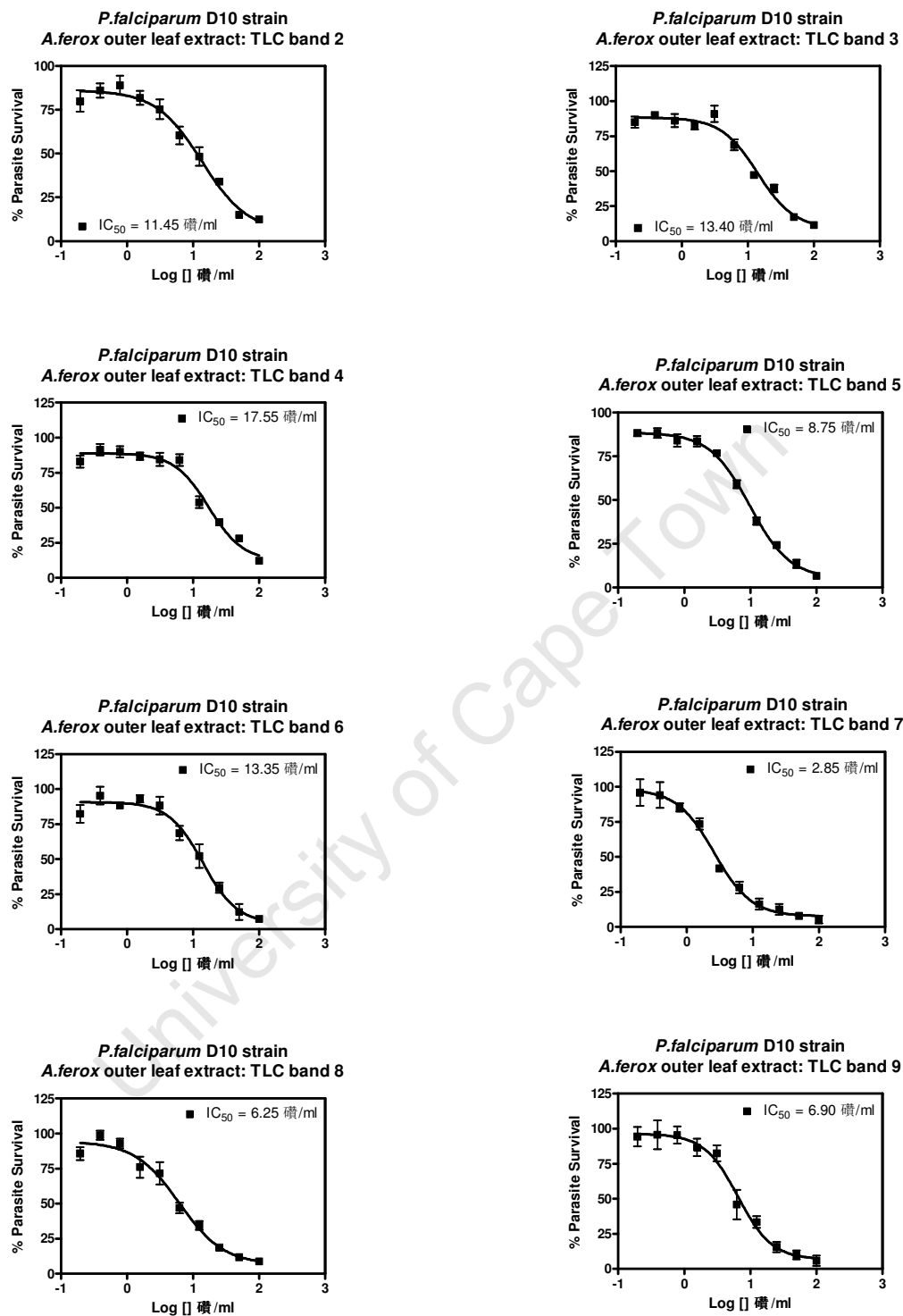


Figure 73 (Continued) Pooled dose-response curves of *A. ferox* extracts separated via TLC tested against the CQS D10 strain of *P. falciparum* (n = 2)

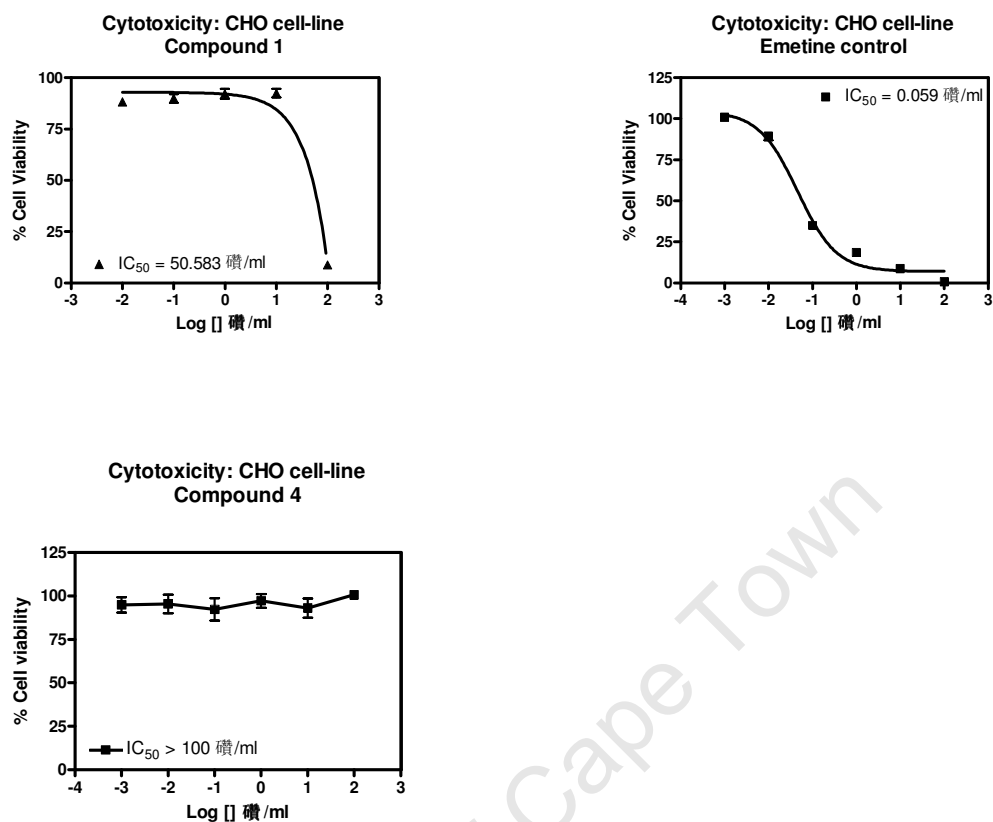


Figure 74 Pooled dose-response curves of compounds isolated from *A. ferox* against the CHO cell-line (n = 2)

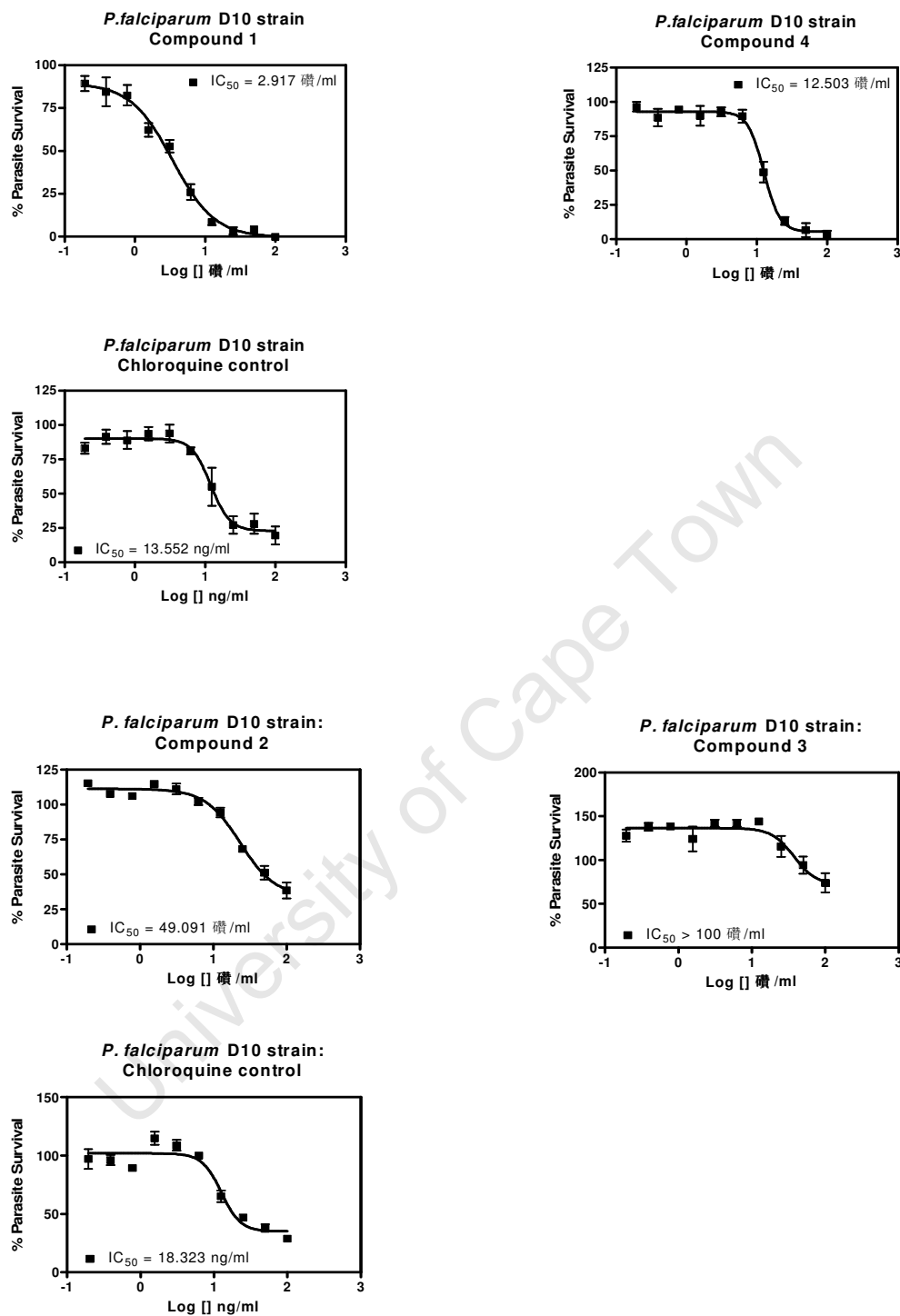


Figure 75 Pooled dose-response curves of compounds isolated from *A. ferox* against the CQS D10 strain of *P. falciparum* (n = 2)

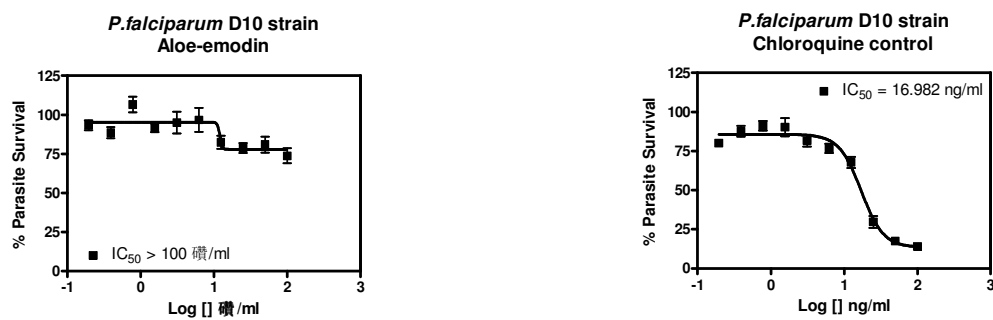


Figure 76 Pooled dose-response curves of Aloe-emodin and chloroquine against the CQS D10 strain of *P. falciparum* (n = 3)

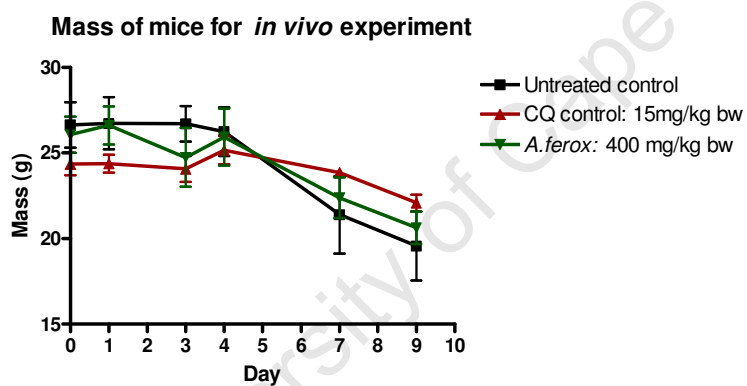


Figure 77 *A. ferox*: Averaged weights of mice using the four-day suppressive test against the CQR *P. yoelii* NS strain

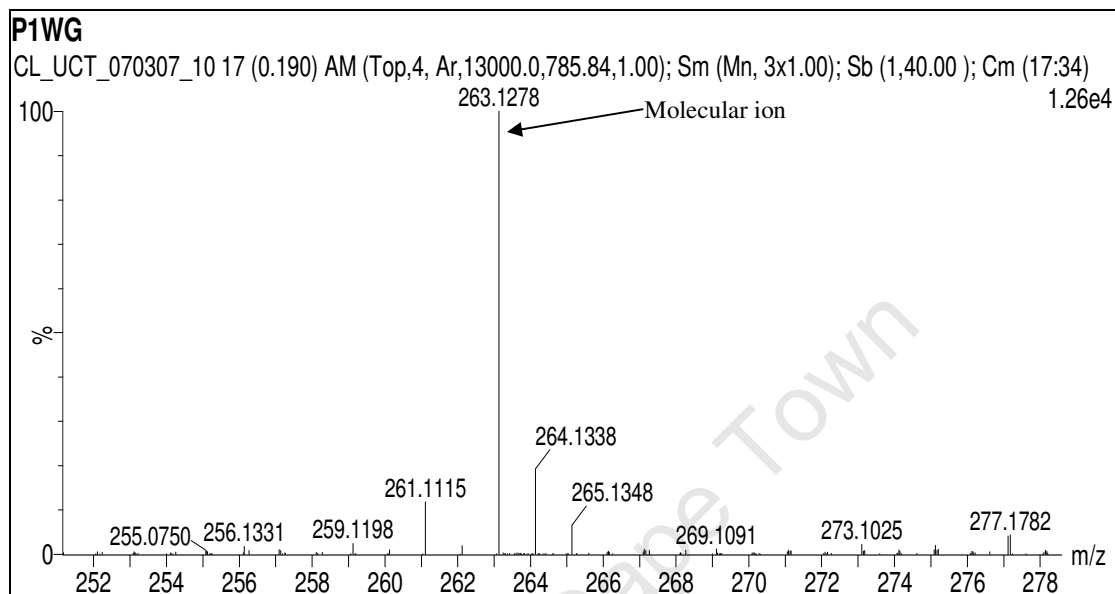
# *Appendix II*

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*MS and NMR spectra*

University of Cape Town

**Mass spectrometry data of compounds 1–3 isolated from  
*S.aethiopicus***



**Elemental Composition**

File Edit View Process Help

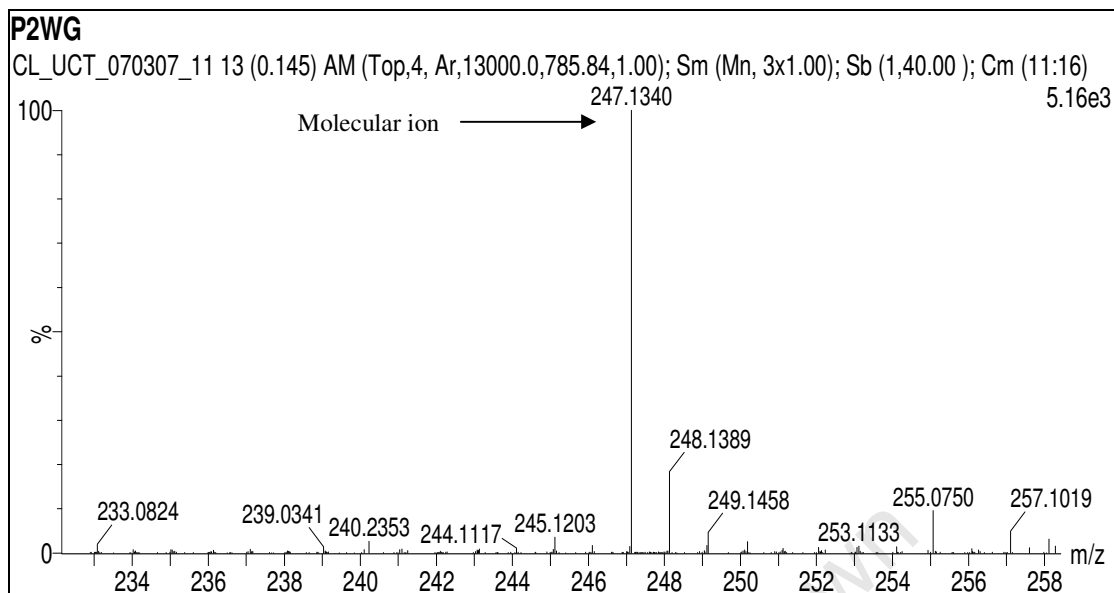
Single Mass Analysis  
Tolerance = 200.0 PPM / DBE: min = -1.5, max = 100.0  
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

**Monoisotopic Mass, Odd and Even Electron Ions**  
9 formula[e] evaluated with 4 results within limits (up to 50 closest results for each mass)

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Score	C	H	N	O
263.1278	263.1283	-0.5	-2.0	6.5	C15 H19 O4	2	15	19	4	
	263.1521	-24.3	-92.5	6.0	C15 H21 N O3	4	15	21	1	3
	263.1647	-36.9	-140.3	5.5	C16 H23 O3	1	16	23		3
	263.1760	-48.2	-183.0	5.5	C15 H23 N2 O2	3	15	23	2	2

Figure 78 LC-MS data of compound 1





**Elemental Composition**

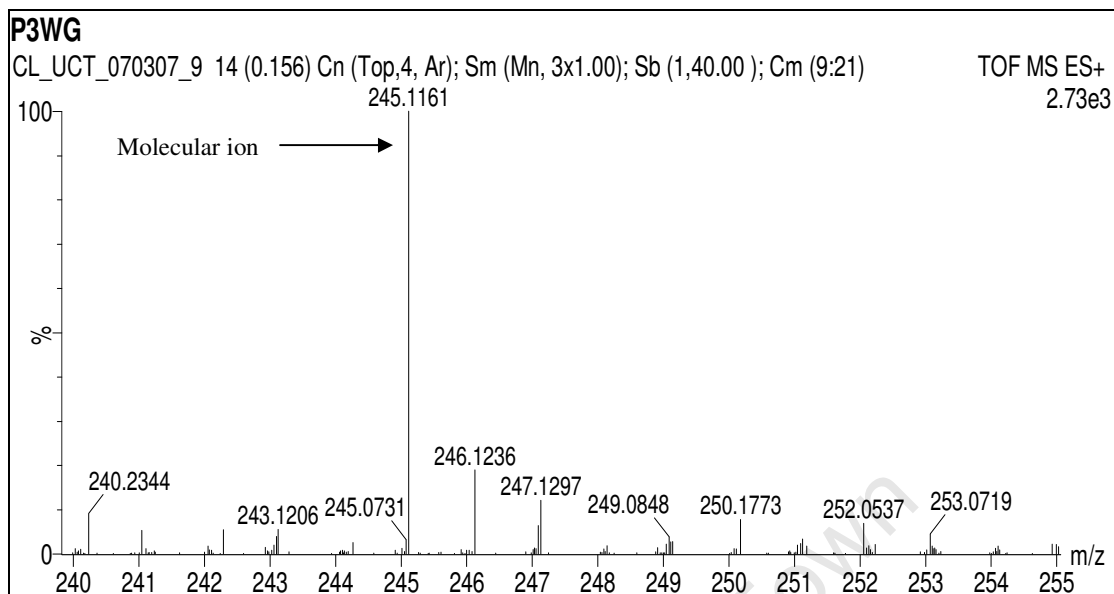
File Edit View Process Help

Single Mass Analysis  
Tolerance = 200.0 PPM / DBE: min = -1.5, max = 100.0  
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

**Monoisotopic Mass, Odd and Even Electron Ions**  
9 formula(e) evaluated with 3 results within limits (up to 50 closest results for each mass)

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Score	C	H	N	O
247.1340	247.1334	0.6	2.3	6.5	C15 H19 O3	2	15	19		3
	247.1572	-23.2	-94.0	6.0	C15 H21 N O2	3	15	21	1	2
	247.1698	-35.8	-144.9	5.5	C16 H23 O2	1	16	23		2

Figure 79 LC-MS data of compound 2



**Elemental Composition**

File Edit View Process Help

Single Mass Analysis  
Tolerance = 200.0 PPM / DBE: min = -1.5, max = 100.0  
Isotope cluster parameters: Separation = 1.0 Abundance = 1.0%

**Monoisotopic Mass, Odd and Even Electron Ions**  
9 formula(e) evaluated with 4 results within limits (up to 50 closest results for each mass)

Mass	Calc. Mass	mDa	PPM	DBE	Formula	Score	C	H	N	O
245.1161	245.1178	-1.7	-6.8	7.5	C15 H17 O3	2	15	17	3	
	245.1290	-12.9	-52.6	7.5	C14 H17 N2 O2	4	14	17	2	2
	245.1416	-25.5	-103.9	7.0	C15 H19 N O2	3	15	19	1	2
	245.1542	-38.1	-155.3	6.5	C16 H21 O2	1	16	21		2

Figure 80 LC-MS data of compound 3

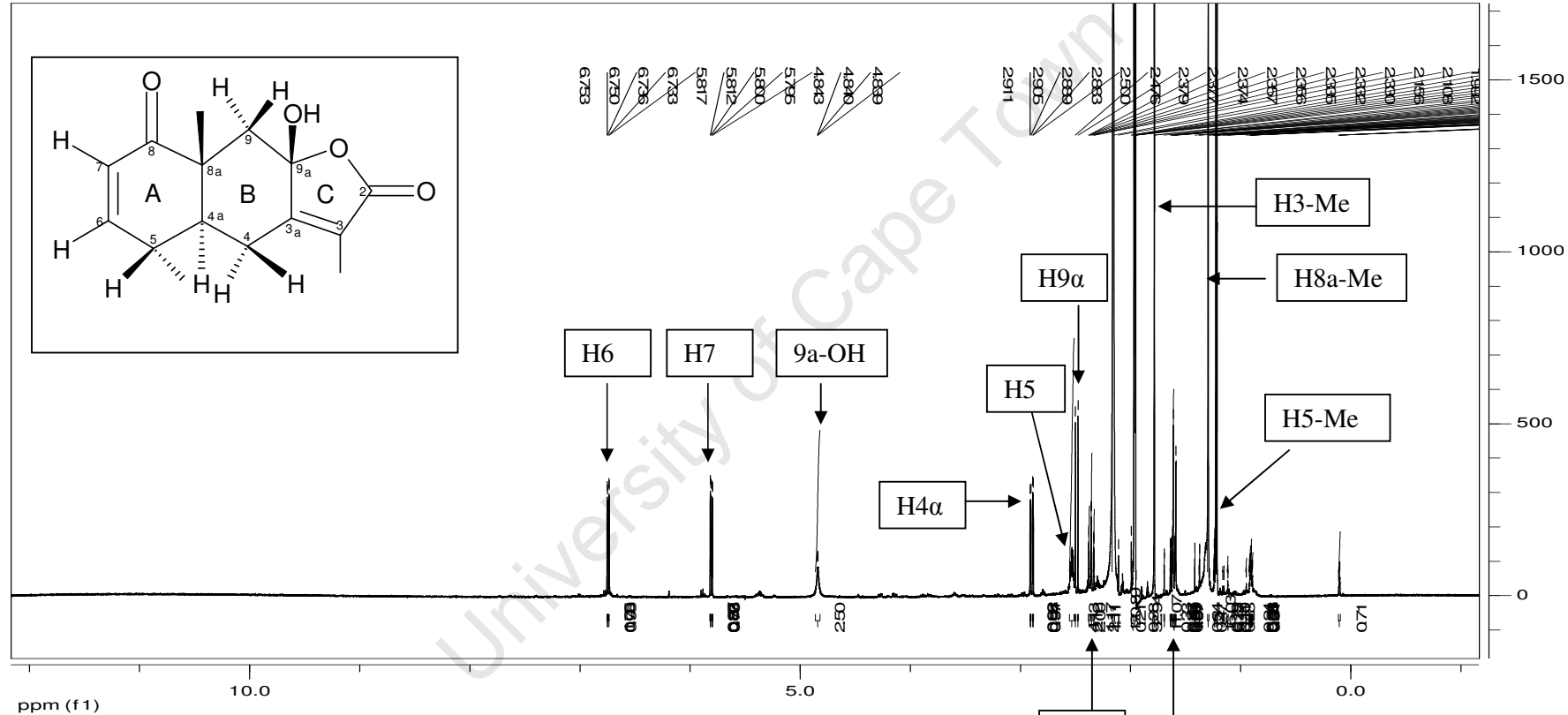
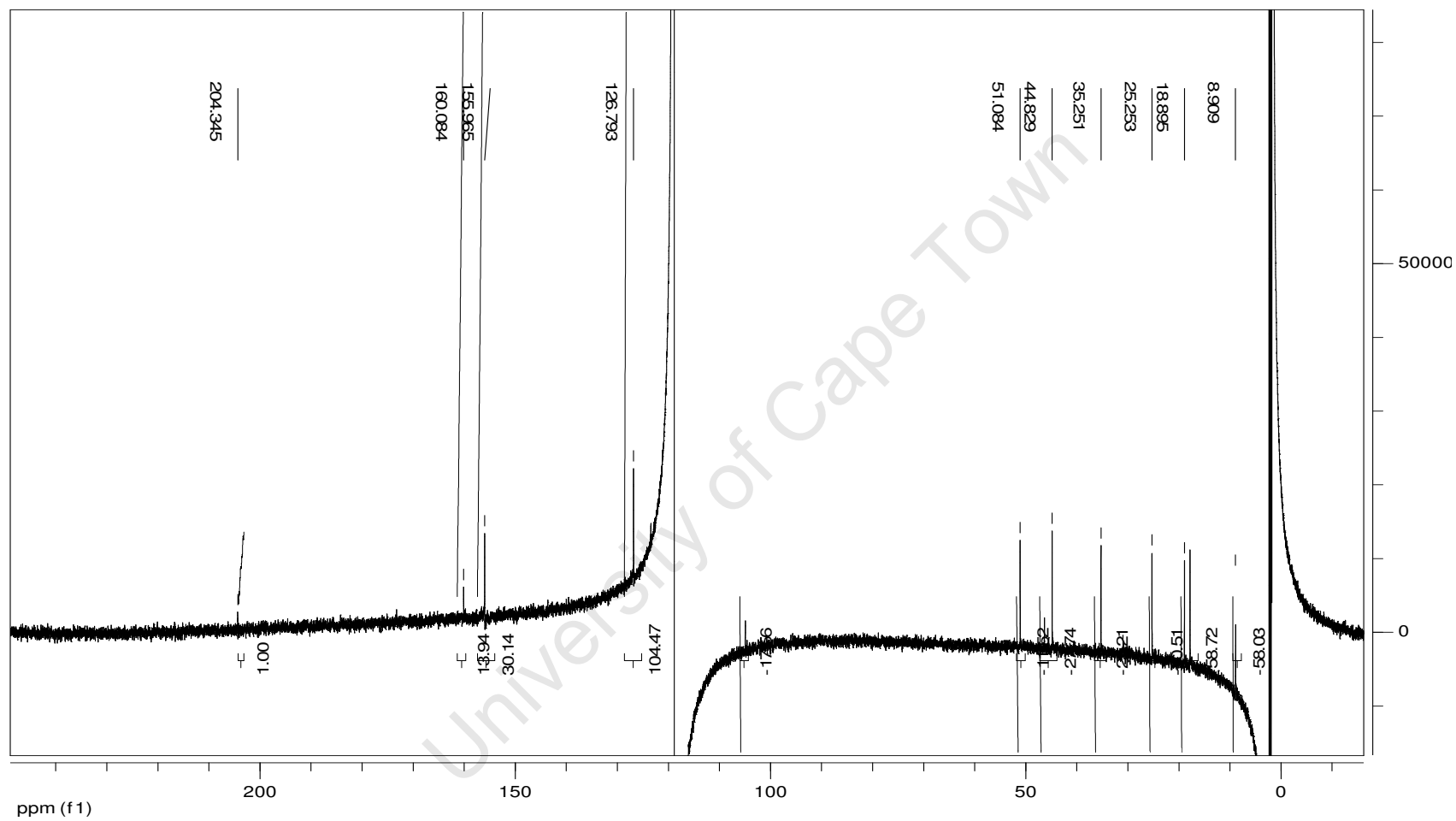


Figure 81  $^1\text{H}$  spectrum of compound 1

Figure 82 <sup>13</sup>C spectrum of compound 1

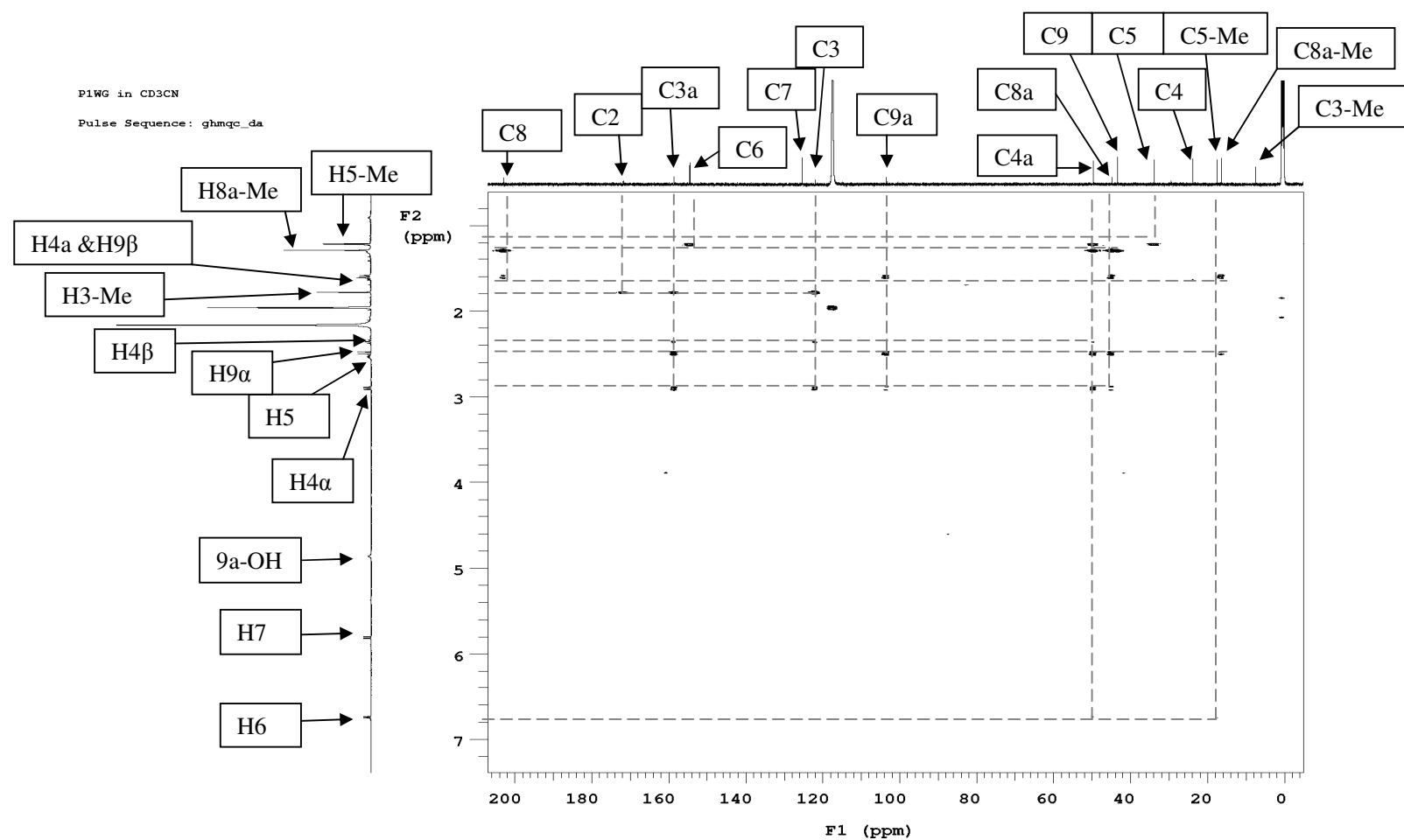


Figure 83 HMQC spectrum of compound 1

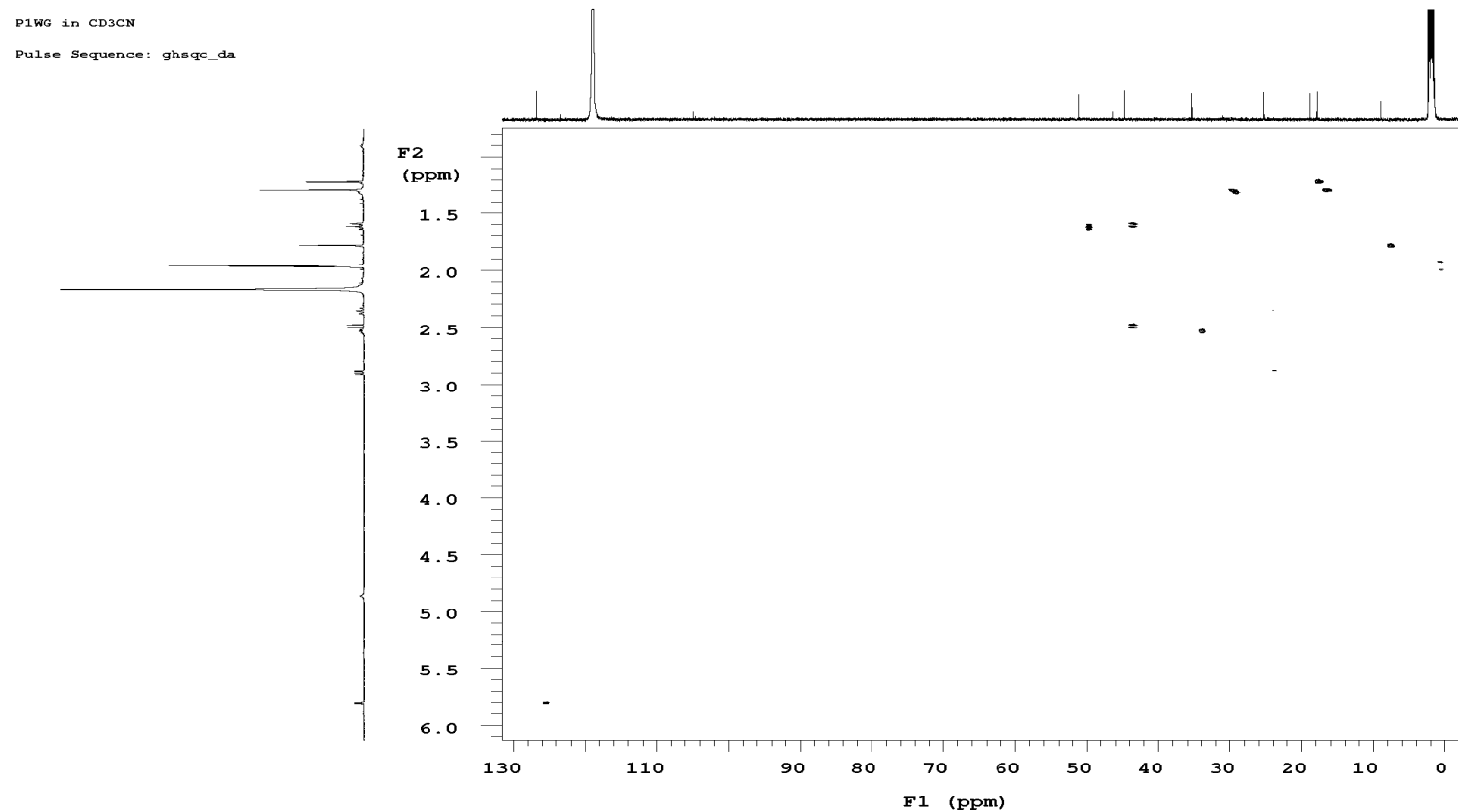


Figure 84 HSQC spectrum of compound 1

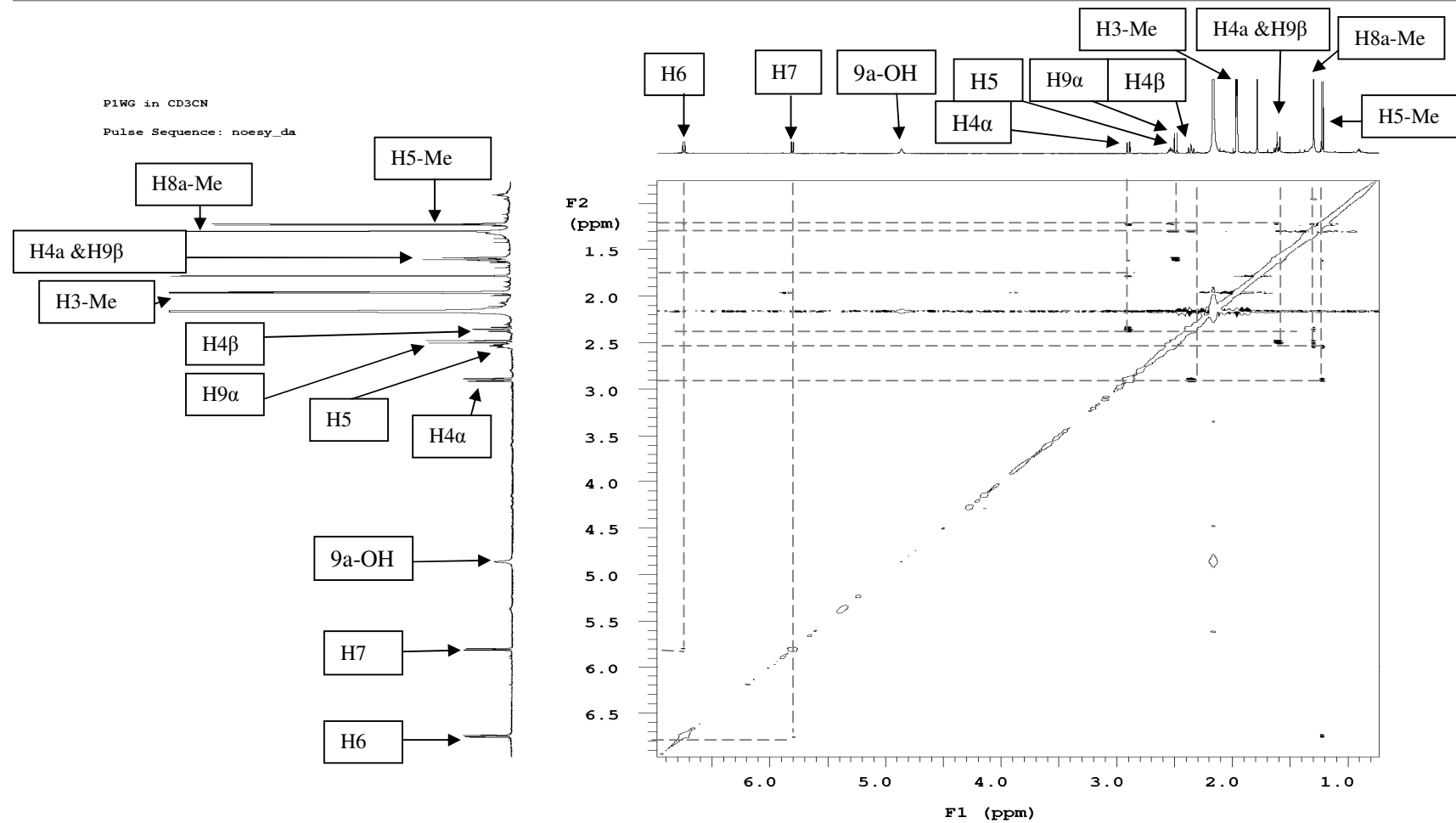


Figure 85 NOESY spectrum of compound 1

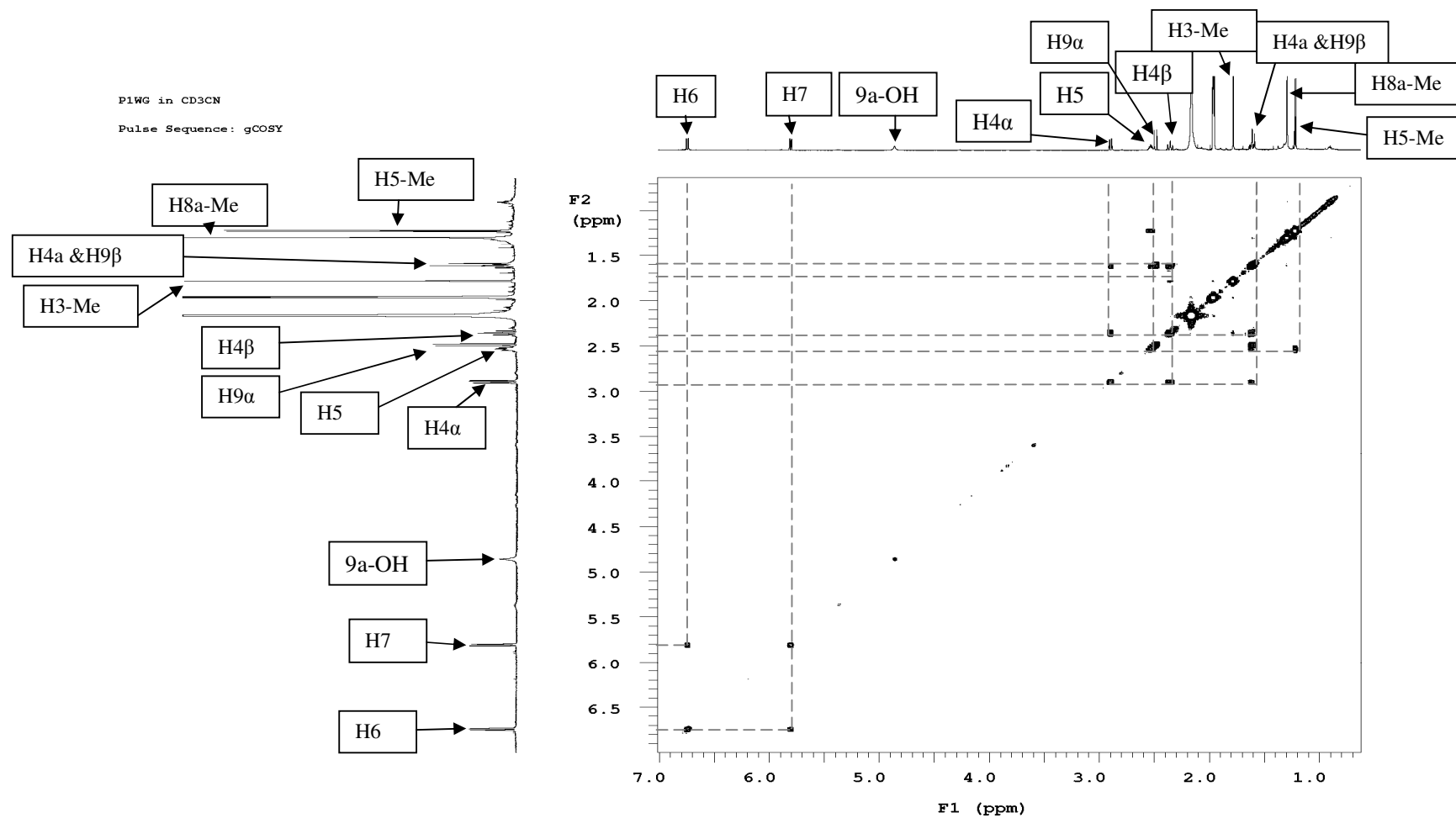
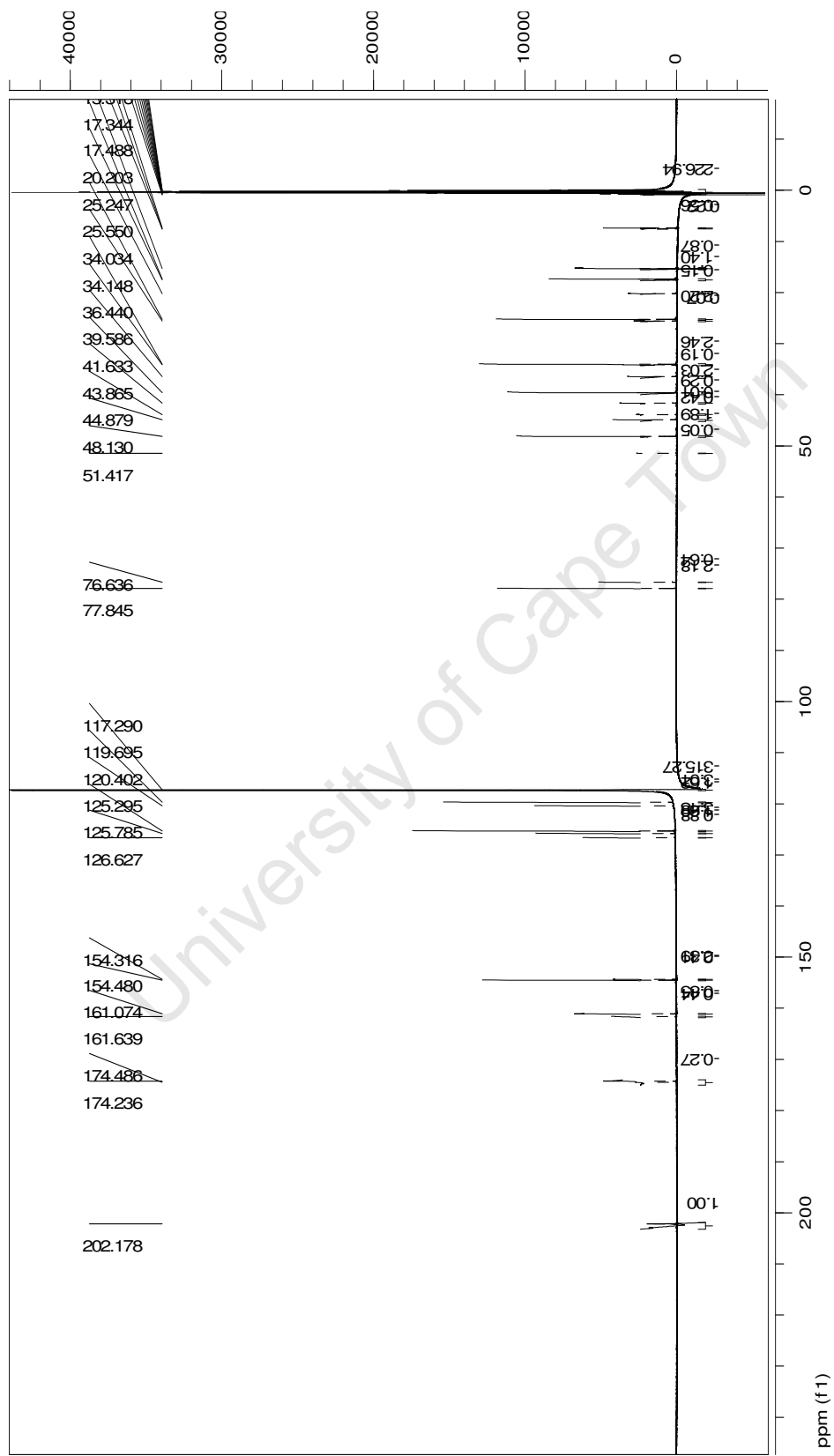


Figure 86 COSY spectrum of compound 1





Figure 88  $^{13}\text{C}$  spectrum of compound 2

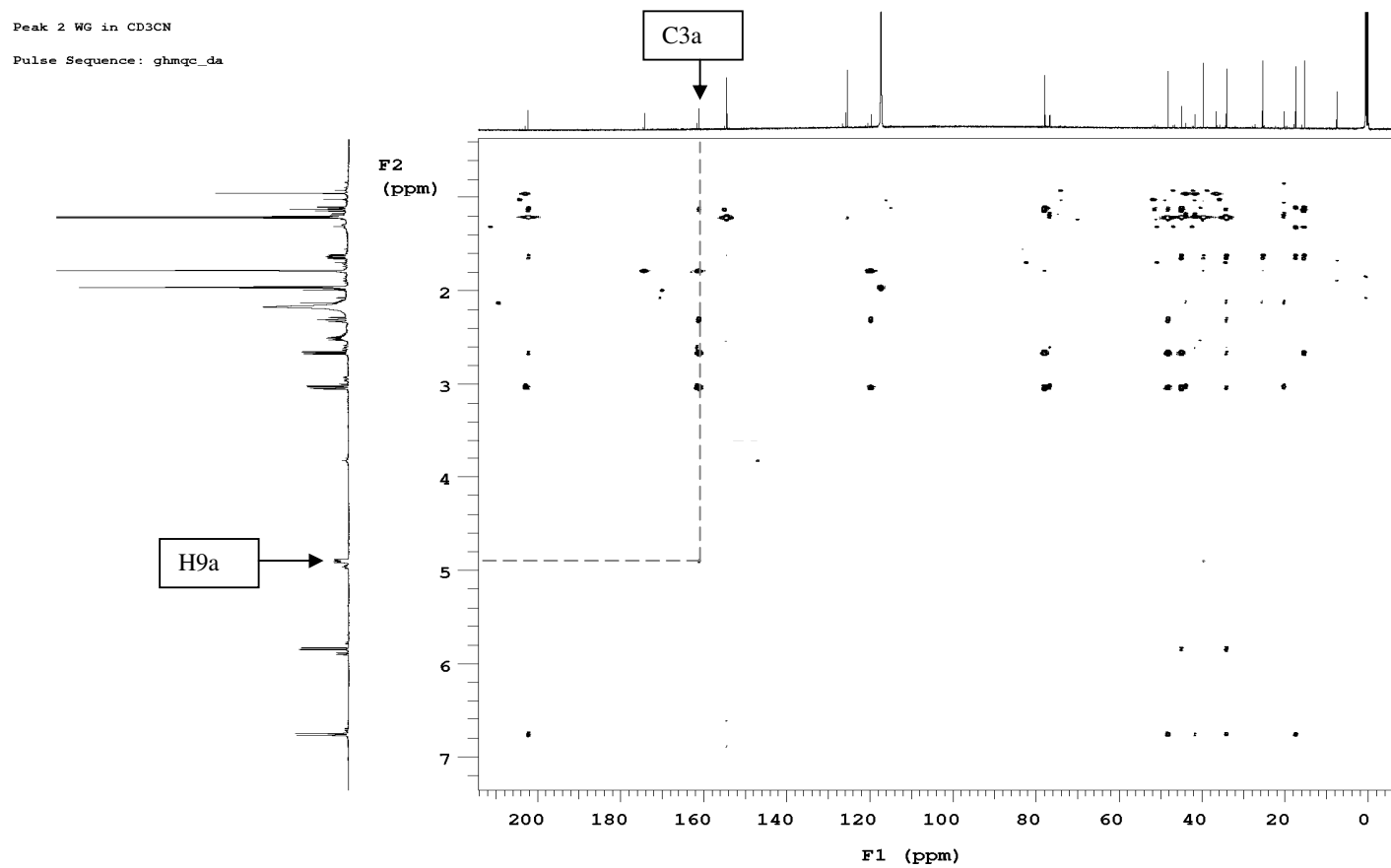


Figure 89 HMQC spectrum of compound 2

## Appendix II

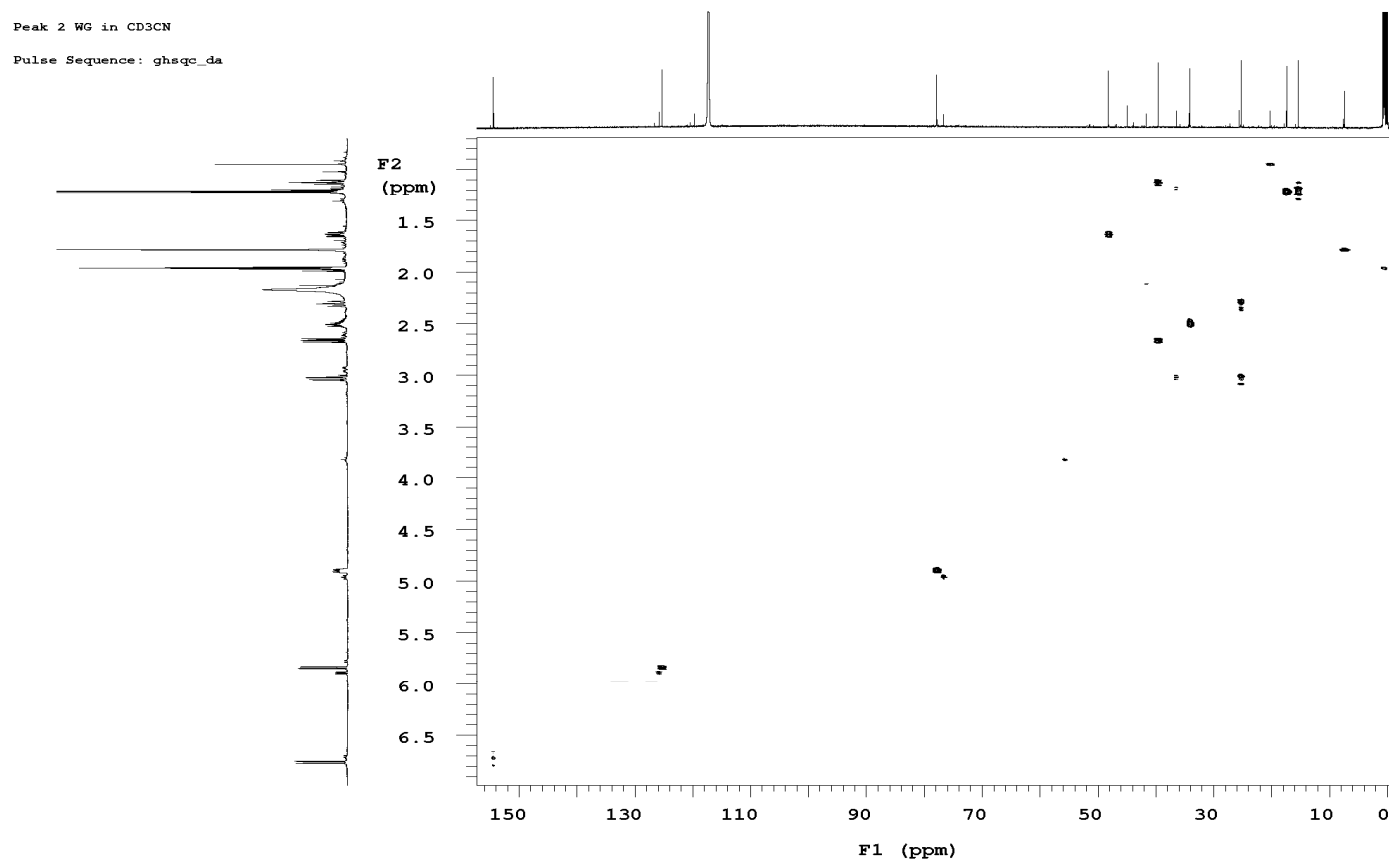


Figure 90 HSQC spectrum of compound 2

## Appendix II

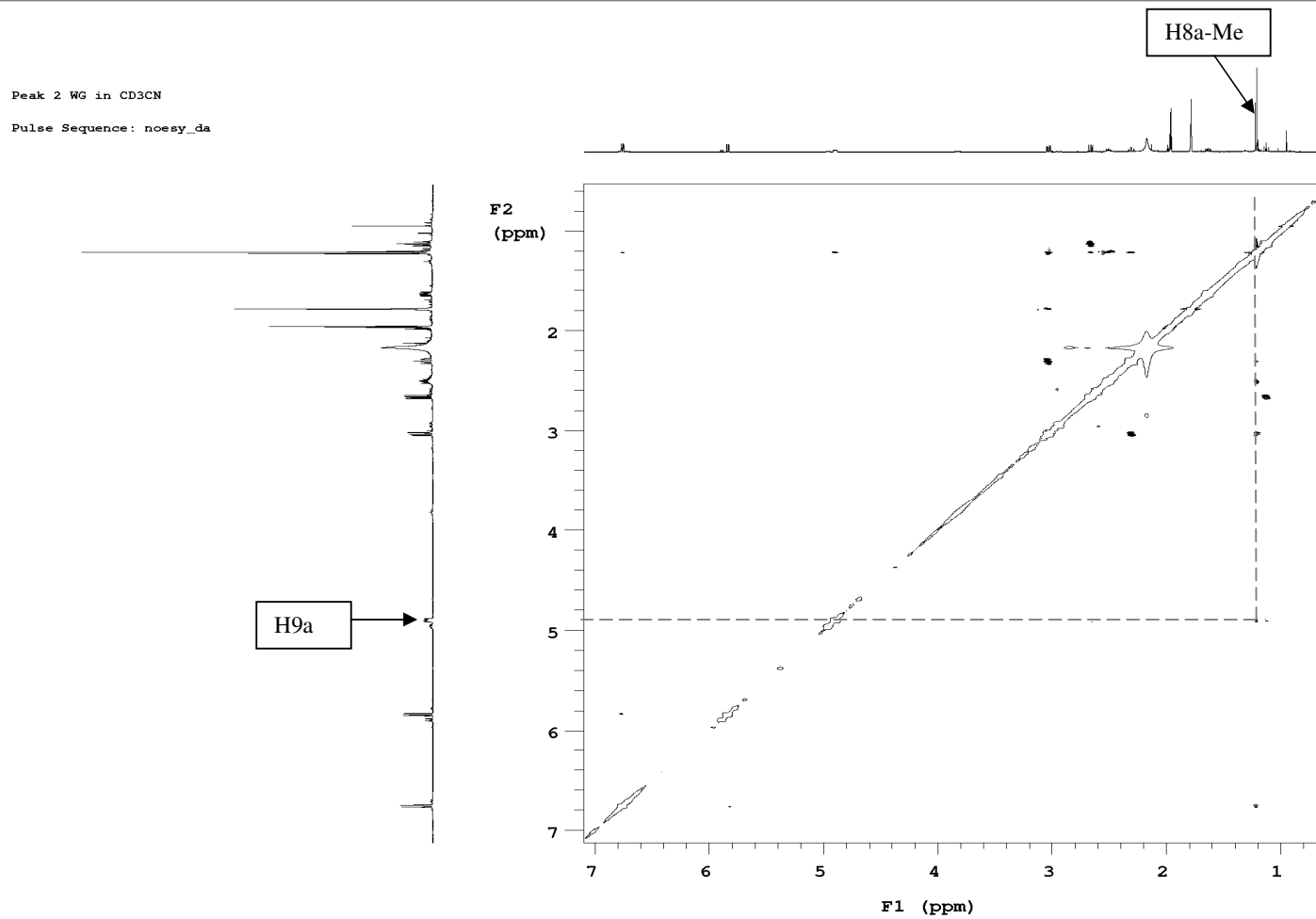


Figure 91 NOESY spectrum of compound 2

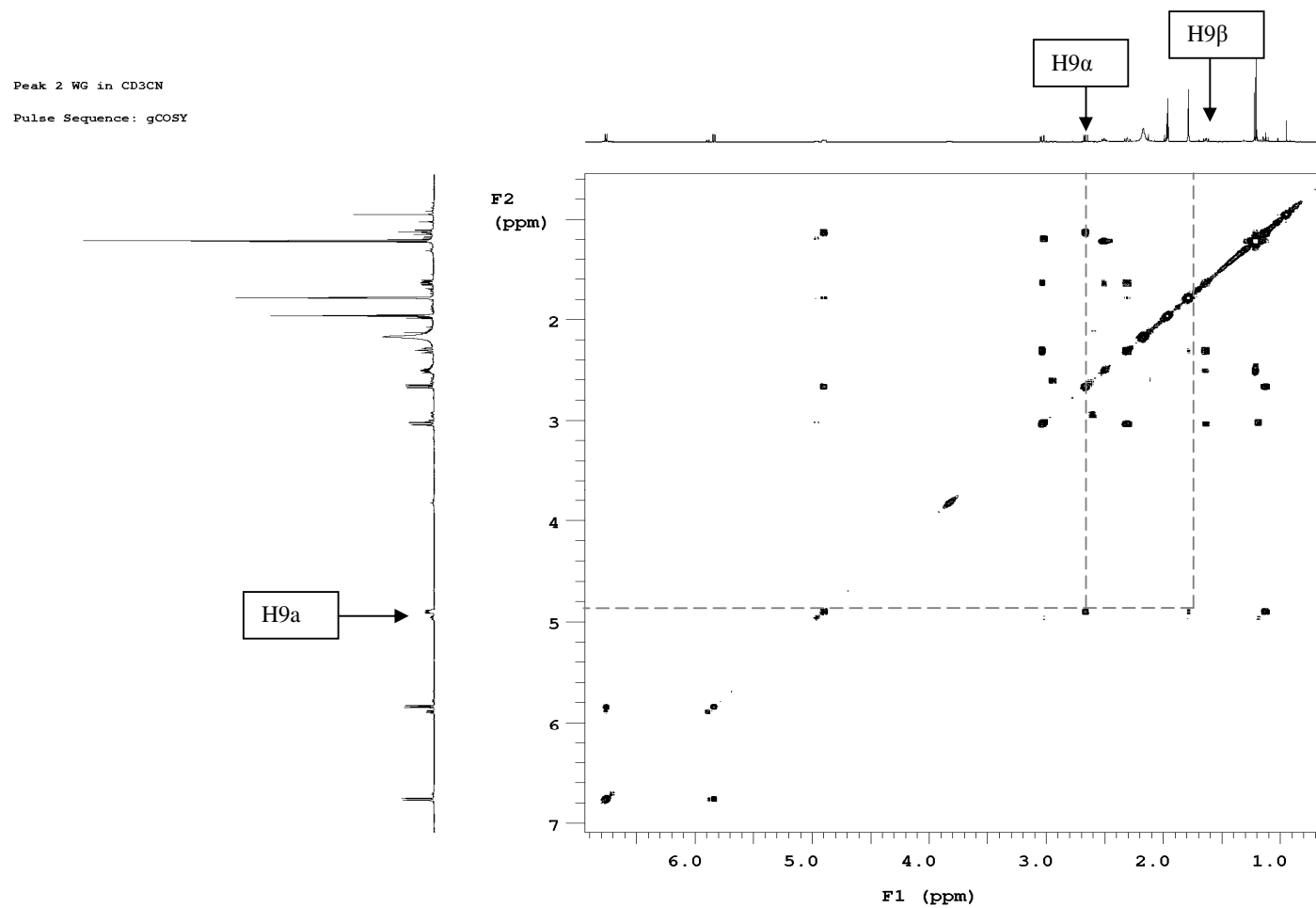
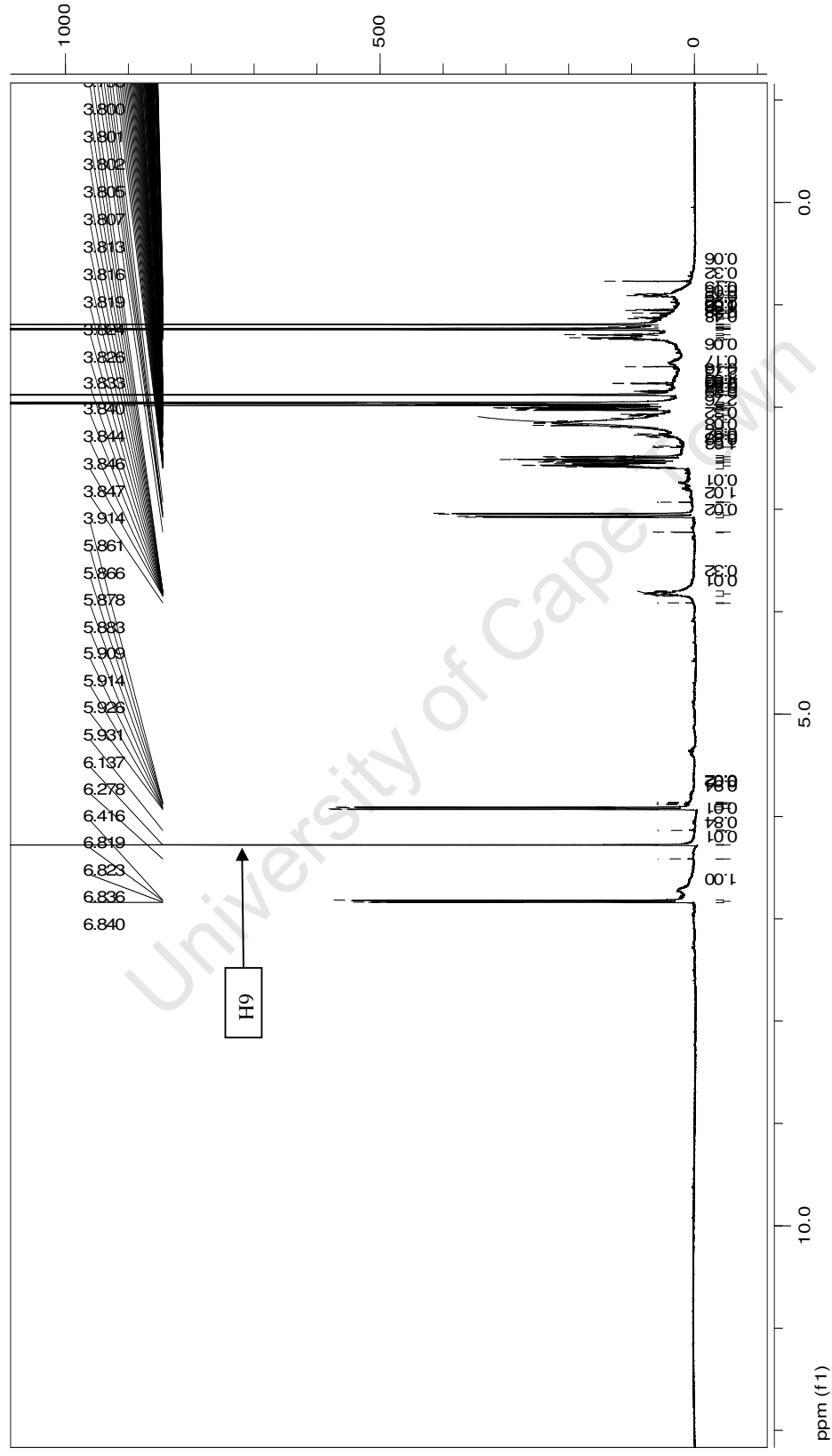
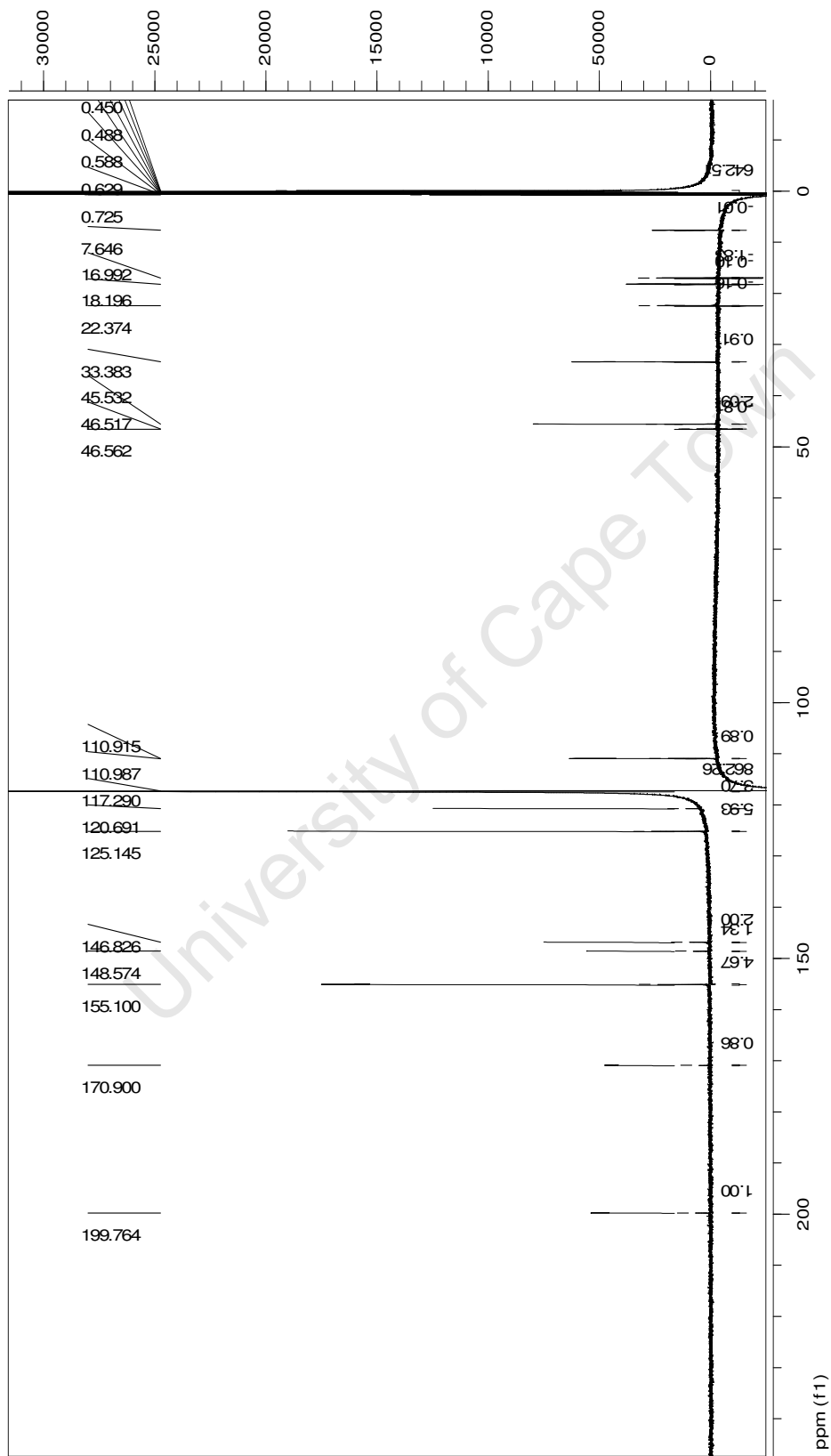


Figure 92 COSY spectrum of compound 2

Figure 93  $^1\text{H}$  spectrum of compound 3

Figure 94  $^{13}\text{C}$  spectrum of compound 3



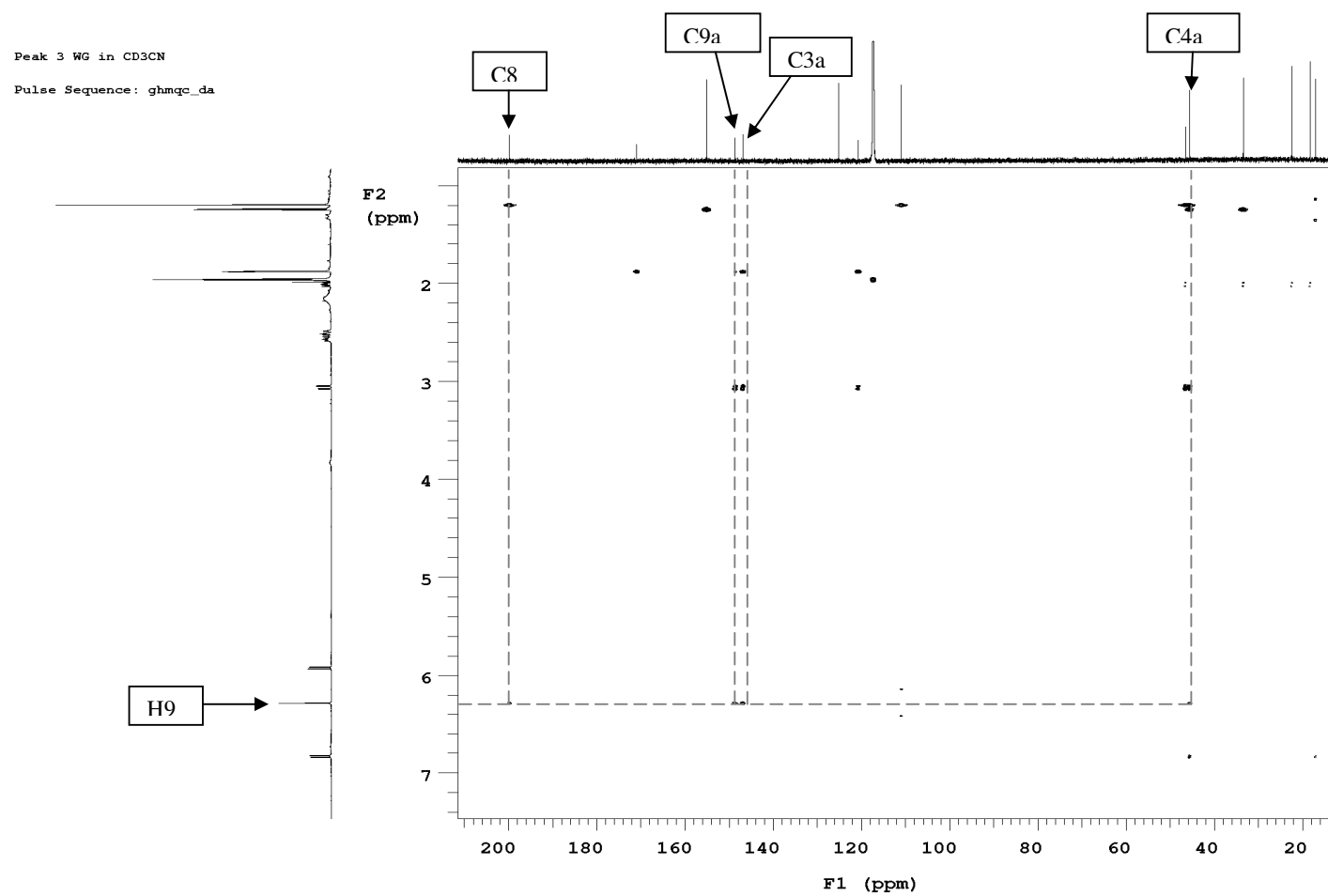


Figure 95 HMQC spectrum of compound 3

## Appendix II

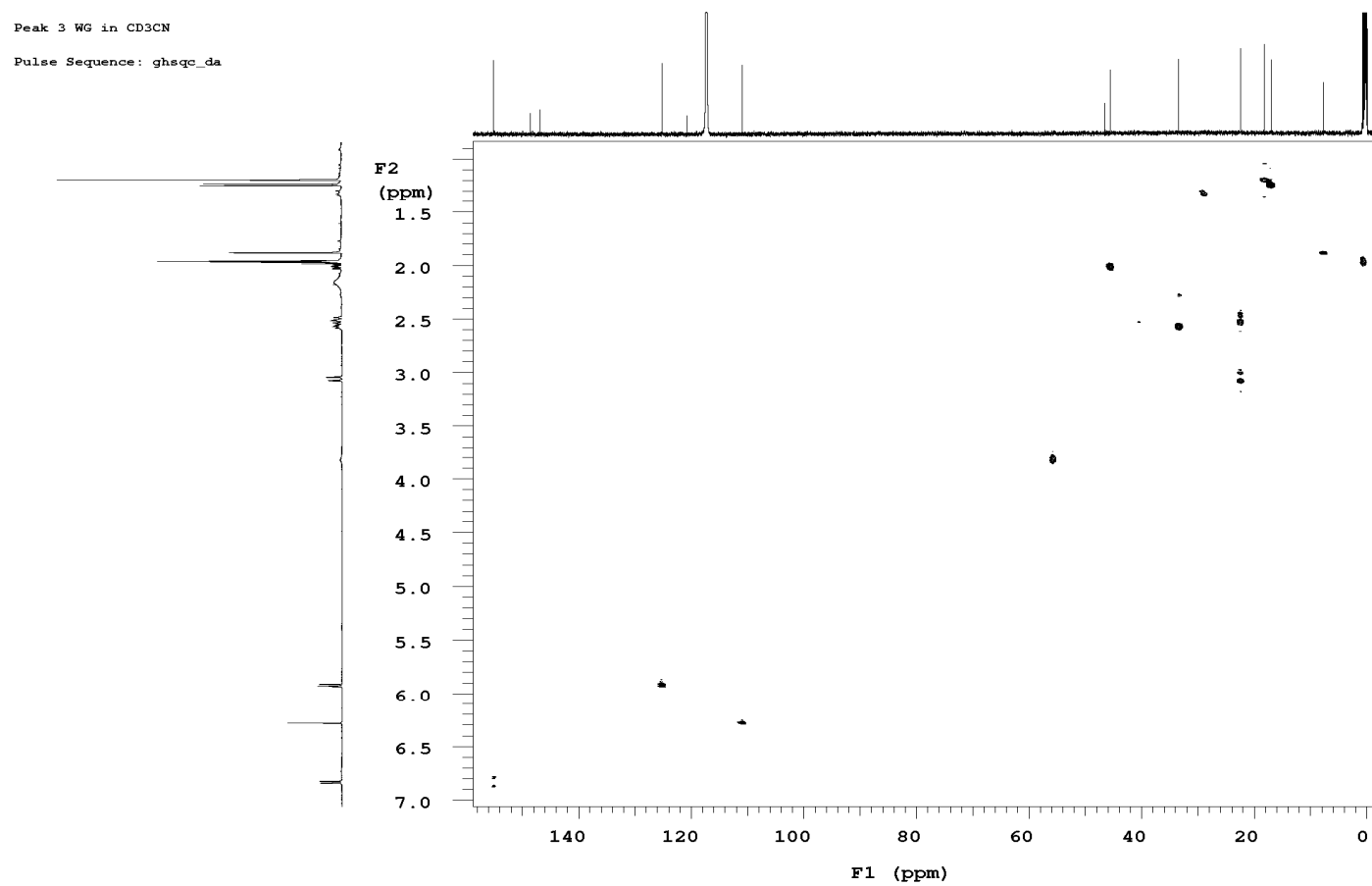


Figure 96 HSQC spectrum of compound 3

## Appendix II

Peak 3 WG in CD<sub>3</sub>CN

Pulse Sequence: noesy\_da

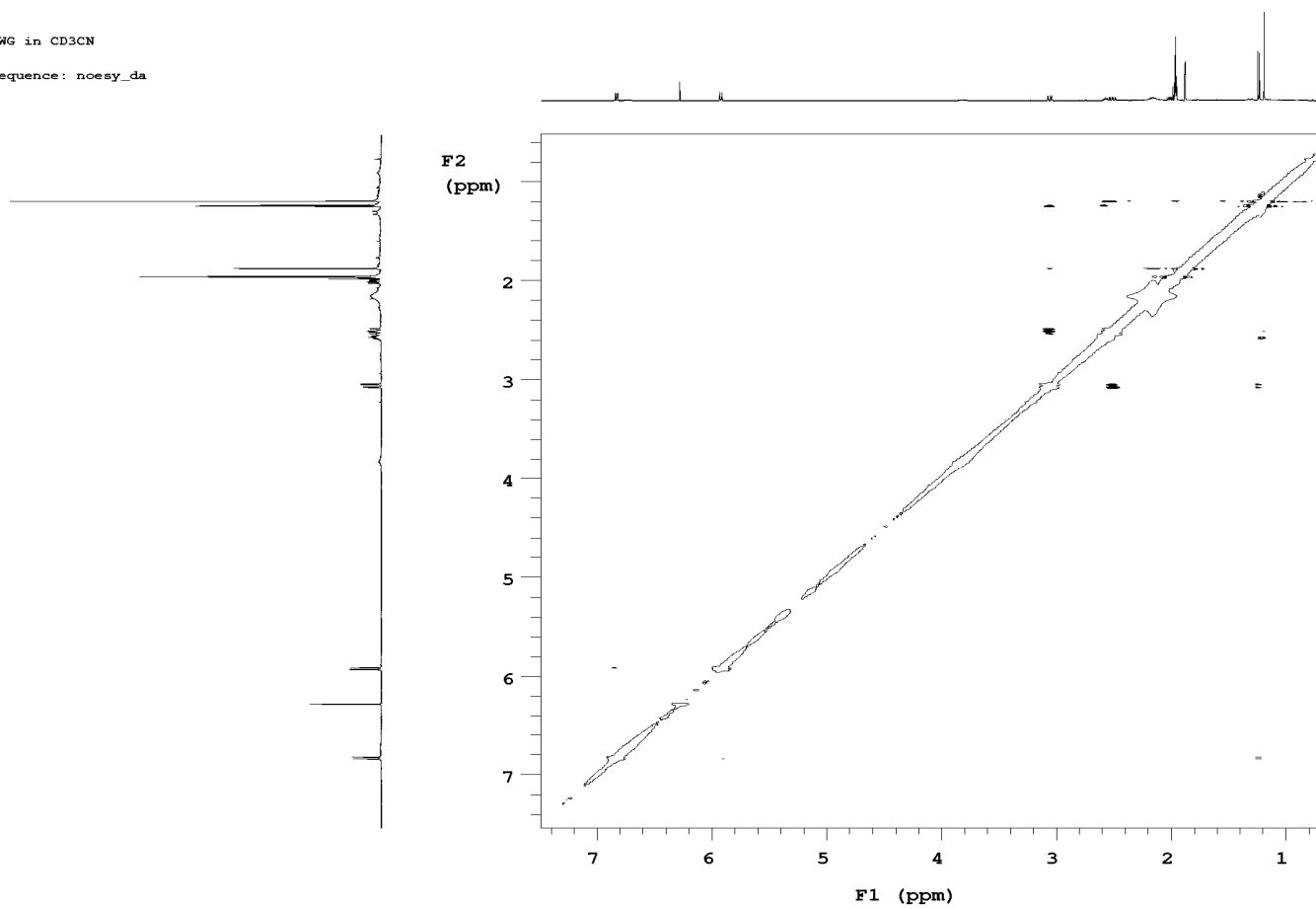


Figure 97 NOESY spectrum of compound 3

## Appendix II

Peak 3 WG in CD<sub>3</sub>CN  
Pulse Sequence: gCOSY

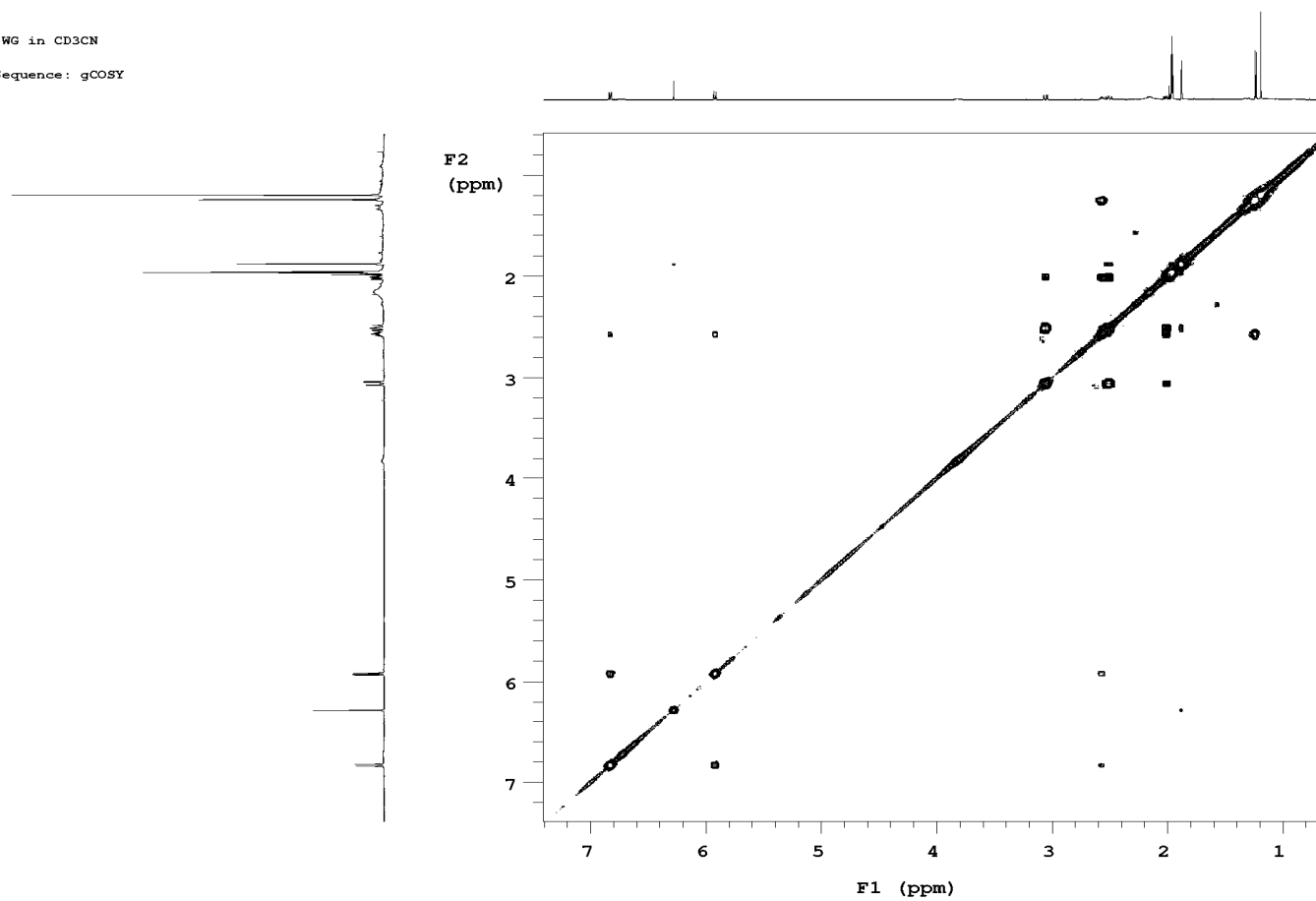


Figure 98 COSY spectrum of compound 3

# *Appendix III*

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*Standard curves for bioavailability study*

University of Cape Town

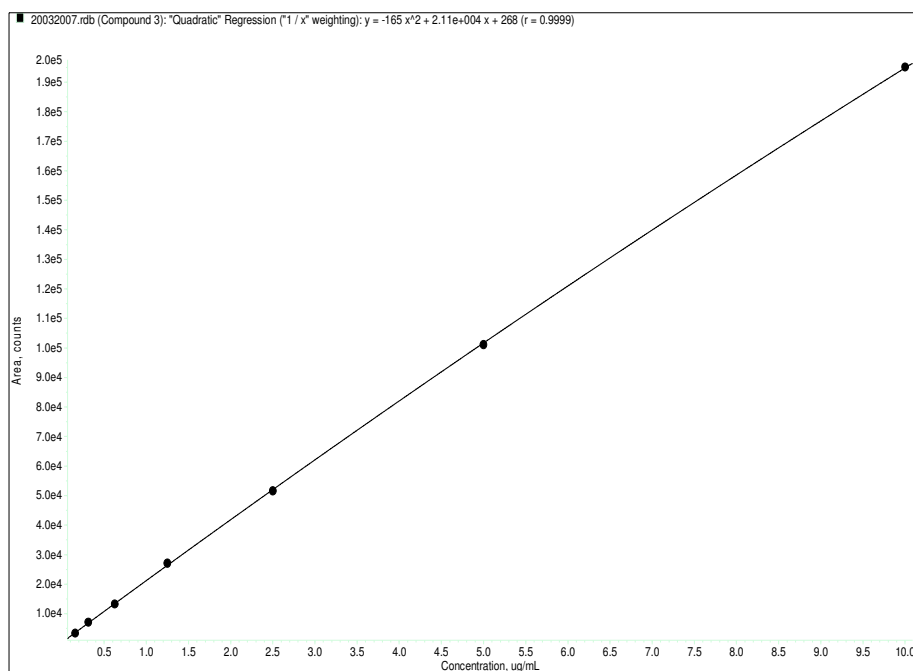
**Standard curves of compound 3 isolated from *S. aethiopicus***

Figure 99 Standard curve for optimisation experiment: pH extractions

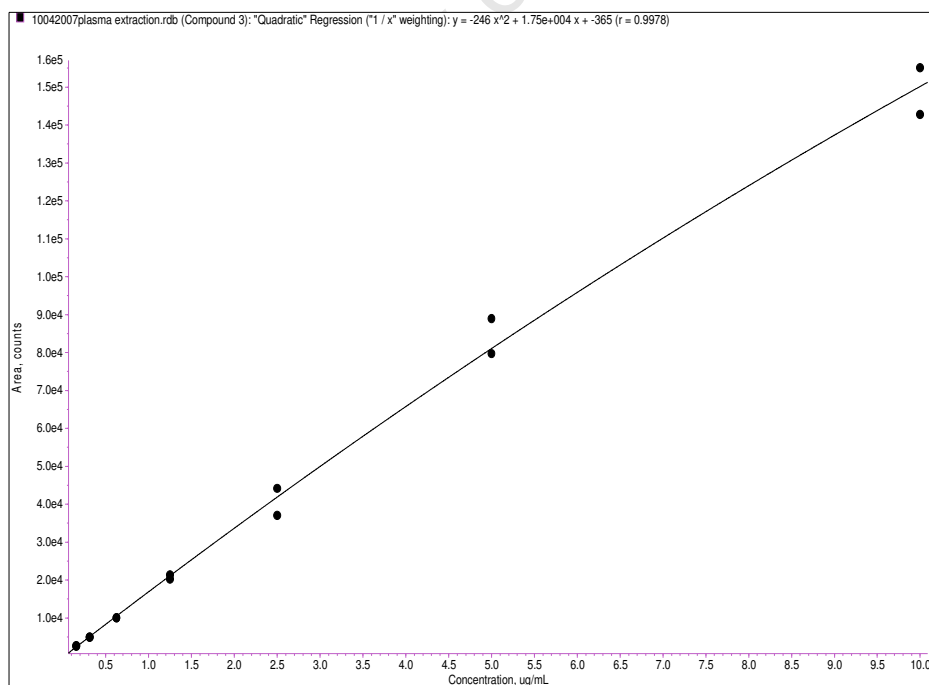


Figure 100 Standard curve for optimisation experiment: Extraction from RBC and plasma

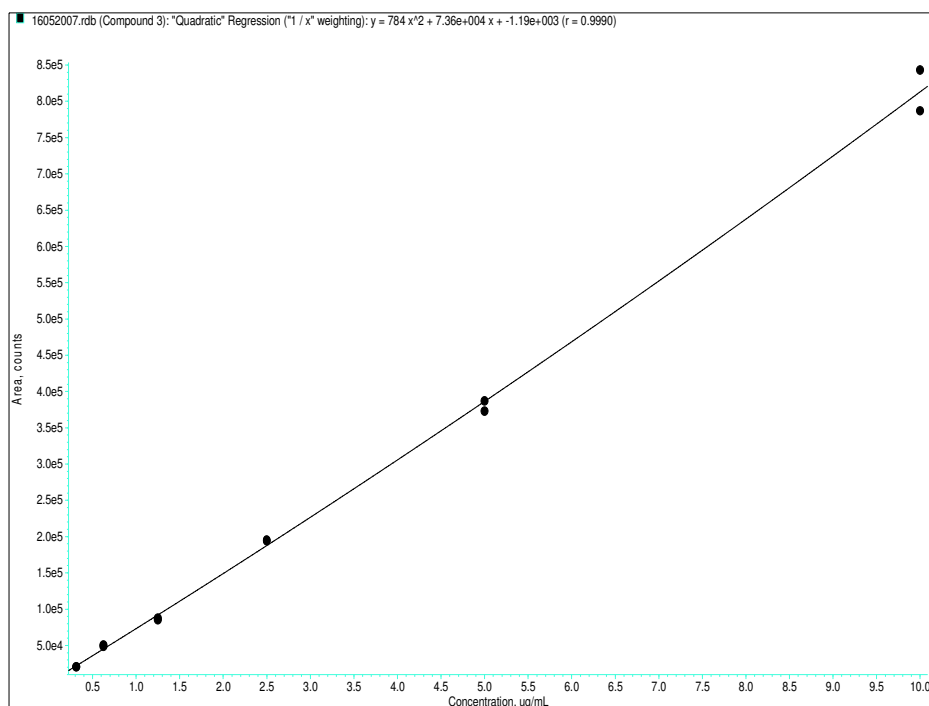


Figure 101 Standard curve for comparative experiment: Oral vs. SC administration of compound 3 in 10% DMSO

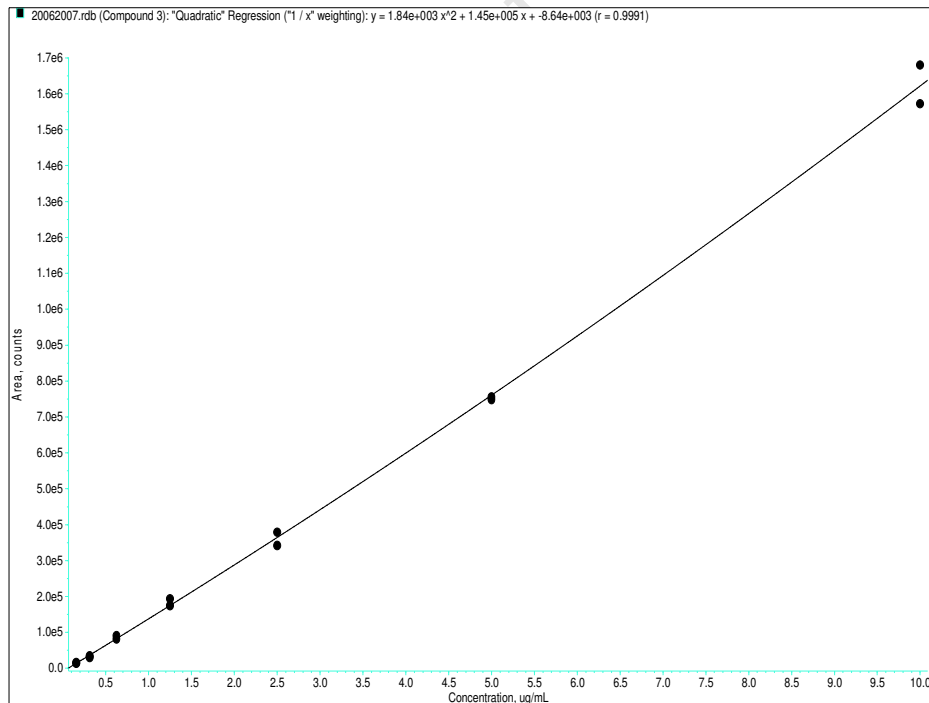


Figure 102 Standard curve for comparative experiment: Oral vs. SC administration of compound 3 in SMEDDS formulation

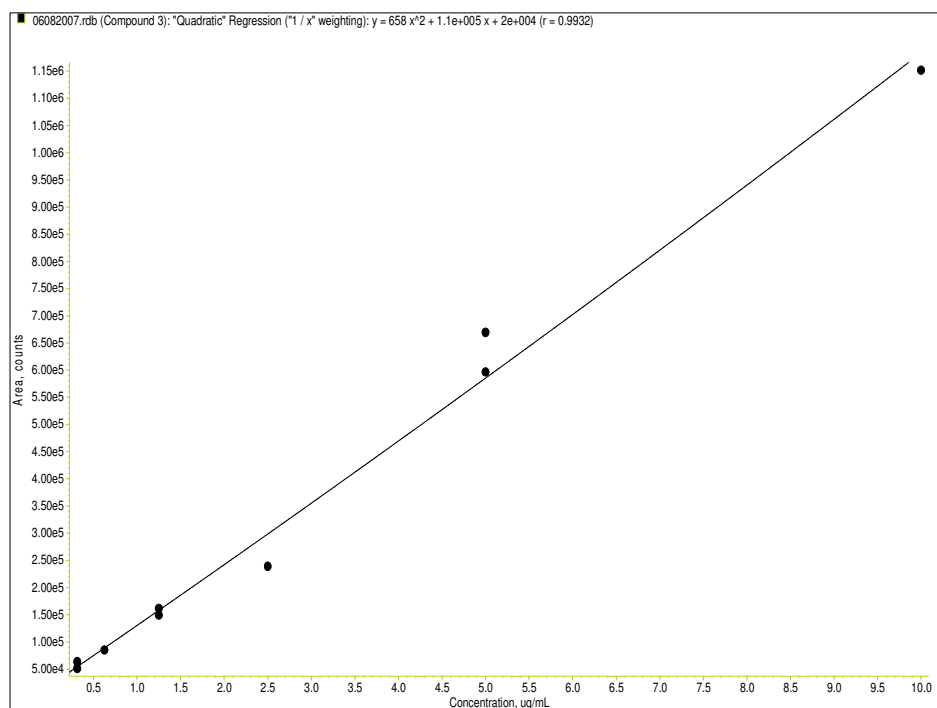


Figure 103 Standard curve for administration of the EA extract of *S. aethiopicus* experiment